444561-08

STUDY TITLE

Validation Report for the Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection

DATA REQUIREMENT

171-4(c) & (d)

AUTHORS

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and
D. R. Foster

STUDY COMPLETED ON

November 24, 1997

PERFORMING LABORATORY

Global Environmental Chemistry Laboratory—Indianapolis Lab
DowElanco
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

RES94084

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Triclopyr

Title: Validation Report for the Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A), (B), or (C).*

Company: DowElanco

Company Agent: S. A. McMaster

Title: Registration Manager

Signature: [signature]

Date: 10/21/97

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Validation Report for the Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection

Study Initiation Date: 01-Jul-1994 Study Completion Date: 24-Nov-1997
Experimental Start Date: 01-Jul-1994 Experiment Termination Date: 06-Jul-1997

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160, except as follows: There was no in-progress audit conducted during the study.

S. A. McMaster
Sponsor
DowElanco

Date 11/21/97

S. A. McMaster
Submitter
DowElanco

Date 11/21/97

E. L. Olberding
Study Director/Author
DowElanco

Date November 24, 1997

DowElanco Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: Triclopyr **Study ID:** RES94084

Title: Validation Report for the Determination of Residues of Triclopyr, 3,5,6-Trichloro~~pyr~~-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection ECB 11/24/97 (TYPO)

Study Initiation Date: 01-Jul-1994 **Study Completion Date:** 24-Nov-1997

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
06-Jun-1994	30-Jun-1994	Protocol Review
17, 19-Nov-1997	20-Nov-1997	Report and Raw Data Audit

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report accurately reflects the raw data generated during the conduct of this study.

Thomas G. Lewis — 11/24/97
Thomas G. Lewis
DowElanco, Quality Assurance

Date

SIGNATURE PAGE

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November 24, 1997
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10/27/97
Date

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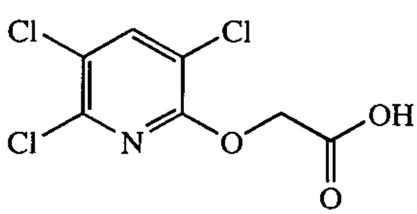
ABSTRACT

This report contains validation data for DowElanco analytical method GRM 97.02. Residues of triclopyr, 3,5,6-trichloro-2-pyridinol, and 2-methoxy-3,5,6-trichloropyridine were determined in fish tissues by capillary gas chromatography with mass selective detection. The method was validated over the concentration range 0.01-5.0 µg/g with a limit of quantitation of 0.01 µg/g.

The mean recovery values and standard deviations of the analytes from samples of fish tissues were 93 ± 6%, 92 ± 9%, and 92 ± 8%, for triclopyr, 3,5,6-trichloro-2-pyridinol, and 2-methoxy-3,5,6-trichloropyridine, respectively. The individual recoveries were all within the acceptable range of 70-120% as specified in the study protocol.

INTRODUCTION

Triclopyr (((3,5,6-trichloro-2-pyridinyl)oxy)acetic acid) is a selective, non-phenoxy herbicide useful in controlling woody plants and broadleaf weeds. Uses of triclopyr-based herbicides include application on rights-of-ways, industrial sites, forest planting sites, turf, range and pasture, and fence rows. The compound is also under development for use as an aquatic herbicide. In the environment, triclopyr degrades primarily to two metabolites, 3,5,6-trichloro-2-pyridinol (3,5,6-TCP), and 2-methoxy-3,5,6-trichloropyridine (2-MP) (1-10). These compounds have also been found in fish and crayfish following exposure to [^{14}C]triclopyr (11-15).



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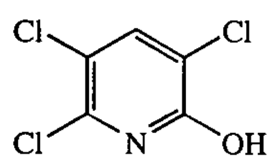
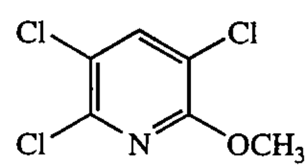
CAS No. 55335-06-3



3,5,6-TCP
CAS No. 6515-38-4



2-MP
CAS No. 31557-34-3

To support existing and proposed registrations, analytical method GRM 97.02 (Appendix A) has been developed for the determination of residues of triclopyr, 3,5,6-trichloro-2-pyridinol, and 2-methoxy-3,5,6-trichloropyridine in fish tissues by capillary gas chromatography with mass selective detection (GC/MSD) (16). The purpose of the study was to provide validation data to define the accuracy, precision, linearity, and specificity, as well as to support the proposed limit of quantitation. The method was validated over the concentration range of 0.01 to 5.0 μg/g with a validated method limit of quantitation of 0.01 μg/g.

EXPERIMENTAL

Sample Origin, Numbering, Preparation, and Storage

Samples of fish tissues were obtained from several sources. The bluegill and catfish samples were collected as part of an aquatic dissipation study (Study Number ENV96052) (17), while the crayfish samples were purchased from Louisiana State University. A portion of these control samples were used for this method validation study.

Upon receipt at DowElanco, the fish tissue samples were inspected and found to be in good condition. Unique sample numbers were assigned to the samples to track them during receipt, storage, and analysis. The fish tissue samples were stored in plastic containers at -15 ° to -20 °C. No significant deviation from this temperature was observed during the storage period between sample receipt and analysis. The sample numbers, sample descriptions, and study numbers are summarized in the table below.

Sample Number	Sample Description	Study Number
SN17993002	bluegill—edible tissue	ENV96052
SN17993003	bluegill—inedible tissue	ENV96052
SN18003302	catfish—edible tissue	ENV96052
SN18003303	catfish—inedible tissue	ENV96052
SN20178201	crayfish—edible tissue	--
SN20178202	crayfish—inedible tissue	--

Analytical Standards

The test substances and analytical standards used in the validation study were the compounds listed in the following table:

Test Substance/ Analytical Standard	AGR/TSN No.	Percent Purity	Certification Date	Reference
triclopyr[a]	TSN100189	99.52	28-Feb-1997	FA&PC 963272
3,5,6-TCP[a]	AGO143197	99.97	01-Mar-1995	ML-AL 94-220167
2-MP[a]	AGR132047	100.0	01-Mar-1995	ML-AL 94-220167
fluroxypyr[b]	AGR222210	99.6	20-Jun-1997	FA&PC 973014
fluroxypyr-DCP[b, c]	AGR251053	99.5	31-Jul-1995	FA&PC 950258
fluroxypyr-MP[b, d]	AGR250194	99.9	24-May-1995	FA&PC 950168

[a] Test substance
[b] Used as internal standard only
[c] 4-amino-3,5-dichloro-6-fluoro-2-pyridinol
[d] 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine

Preparation of Triclopyr, 3,5,6-TCP, and 2-MP Spiking Solutions/Calibration Standards

The triclopyr, 3,5,6-TCP, and 2-MP spiking solutions and calibration standards were prepared as described in method GRM 97.02, Section G.1.

Fortification of Recovery Samples

Untreated control samples were fortified with the mixed triclopyr spiking solutions for the generation of method validation recovery data. On the day of analysis, fish tissue samples were fortified by adding 100 µL of the appropriate fortification solution to 1.0 g of sample. Reagent blank and control samples, containing no mixed triclopyr standard, were also prepared for analysis with the recovery samples to check for background interferences.

Sample Extraction and Analysis

Samples were prepared and extracted as described in Section I.1. of analytical method GRM 97.02 with no modifications. Residues of triclopyr, 3,5,6-TCP, and 2-MP were extracted from fish tissues and hydrolyzed using aqueous 0.25 N sodium hydroxide. Following hydrolysis, the sodium hydroxide was acidified and the analytes extracted with ethyl ether. The ethyl ether was passed through an alumina solid-phase extraction (SPE) column which retained the triclopyr and 3,5,6-TCP, while the 2-MP was contained in the ethyl ether eluate. For the determination of 2-MP, the ethyl ether was concentrated and exchanged with 1-chlorobutane. The triclopyr and 3,5,6-TCP were eluted from the alumina column with 0.1 N sodium hydroxide, which was subsequently acidified and purified using a C_{18} SPE procedure. The eluate from the C_{18} SPE was then extracted with 1-chlorobutane. For both sample fractions, the 1-chlorobutane was concentrated to less than 1 mL, and an acetone solution containing fluroxypyr analogs as internal standards was added. The samples were then derivatized with *N*-methyl-*N*-(*tert*-butyldimethyl-silyl)-trifluoracetamide (MTBSTFA) to form the *tert*-butyldimethylsilyl (TBDMS) derivatives of triclopyr and 3,5,6-TCP. The samples were then analyzed by capillary GC/MSD.

Calculation of Percent Recovery

Calculation of percent recovery for fortified samples was performed as described in method GRM 97.02, Section I.2.

Statistical Treatment of Data

Statistical treatment of data included the calculation of the least squares regression equations and the correlation coefficients for describing the linearity of the calibration curves, and the calculation of the means, standard deviations, and the relative standard deviations of the results for the fortified recovery samples

Study Personnel

The following personnel were involved in various aspects of conducting DowElanco Study Number RES94084: E. L. Olberding, D. R. Foster, M. A. Bartels, D. A. McNett, and R. D. Griggs.

RESULTS AND DISCUSSION

Radiolabeled Extraction Efficiency Study

For this method validation study, no new extraction efficiency studies were conducted using radiolabeled analytes. In previous work (11-14), triclopyr, 3,5,6-TCP, 2-MP and their conjugates were shown to be the major compounds found in fish tissues following exposure to [^{14}C]triclopyr. Various extraction solvents and regimens were used in these studies to completely recover the radioactivity from the samples. In the most recent of these studies (15), fish tissues heated with aqueous sodium hydroxide for 2 hours at 80 °C were found to extract and hydrolyze the ^{14}C-labeled residues. This extraction procedure was adopted in the present methodology.

Analytical Recovery Data

The method validation study was conducted to determine the recovery levels and the precision of the method for the determination of residues of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues. The results are summarized in method GRM 97.02, Tables I-III.

Recovery values of triclopyr from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 μg/g averaged 93% with one standard deviation equal to 6% (Table I). Recovery values of 3,5,6-TCP from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 µg/g averaged 92% with one standard deviation equal to 9% (Table II). Recovery values of 2-MP from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 µg/g averaged 92% with one standard deviation equal to 8% (Table III). The individual recoveries were all within the acceptable range of 70-120% as specified in the protocol.

Calculated Limits of Quantitation and Detection

Following established guidelines (18), the limits of quantitation (LOQ) and detection (LOD) were calculated using the standard deviation from the 0.01-µg/g recovery results. The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the results of the analysis of twelve samples. The results are tabulated in method GRM 97.02, Tables I-III.

The calculated LOQ ranged from 0.007 to 0.009 µg/g for the three analytes, which is lower than the targeted method LOQ of 0.01 µg/g. Results should not be quantified, however, at levels below which no recovery samples have been analyzed.

In a similar fashion, the calculated LOD ranged from 0.002 to 0.003 µg/g for the three analytes. However, since the lowest level of fortification for recovery samples was 0.003 µg/g, the method LOD is considered to be 0.003 µg/g.

Confirmation of Residue Identity

Confirmation of the presence of residues of the analytes is described in method GRM 97.02, Sections I.2.b. and K.2. For the three analytes, confirmation is by comparison of the retention time (gas chromatography) as well as the peak area ratios resulting from selected ion monitoring (mass spectrometry). Confirmation of the presence of the analytes is indicated when the retention times match those of the standards and the confirmation ratio is in the range of ±20% of the average found for the standards. If additional confirmation is required beyond that discussed in this method, the mass spectra of triclopyr-TBDMS and triclopyr-MP contain additional ions that may be used for confirmation. The confirmation ratios of the samples analyzed during the method validation all fell within ±10% of the average found for the calibration standards.

Representative Full-Scan Mass Spectra

Typical full-scan mass spectra of the TBDMS derivatives of triclopyr and 3,5,6-TCP, and 2-MP are shown in method GRM 97.02, Figures 3-5. Typical full-scan mass spectra of the TBDMS derivatives of fluroxypyr and fluroxypyr-DCP, and fluroxypyr-MP are shown in method GRM 97.02, Figures 3-5.

Representative Calibration Curves

Typical calibration curves for the determination of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues are shown in method GRM 95.19, Figures 6-8.

Representative Chromatograms

Typical chromatograms of a standard, control sample, and a 0.01-µg/g recovery sample for the determination of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues are illustrated in method GRM 97.02, Figures 9-17, respectively. None of the control samples in the method validation study contained interference peaks at the retention times of the analytes or internal standards.

Representative SPE Elution Profiles

Typical elution profiles for 2-MP, triclopyr, and 3,5,6-TCP on an alumina SPE column are illustrated in method GRM 97.02, Figure 18. Typical elution profiles for triclopyr and 3,5,6-TCP on a C_{18} SPE column are illustrated in method GRM 97.02, Figure 19.

CONCLUSIONS

DowElanco analytical method GRM 97.02, "Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection", has been demonstrated to be suitable for the determination of these analytes in fish tissues. The method was validated over the concentration range of 0.01 to 5.0 μg/g with a validated method LOQ of 0.01 μg/g. The individual recoveries for each of the fortified samples were all within the acceptable range of 70-120% as specified in the study protocol.

ARCHIVING

The protocol, raw data, and final report are all filed in the DowElanco testing facility archives at 9330 Zionsville Road, Indianapolis, Indiana 46268-1054.

ACKNOWLEDGEMENTS

Initial sample documentation, handling, and preparation were conducted by R. D. Griggs.

REFERENCES

1. Bidlack, H. D.; Laskowski, D. A.; Swann, R. L.; Comeaux, L. B.; Jeffries, T. K. "Comparison of the Degradation Rates and Decomposition Products of ^{14}C-Triclopyr in Aerobic and Waterlogged Soil", GH-C 919 (Revised), 1977, unpublished report of DowElanco.

2. McCall, P. J.; Laskowski, D. A.; Jeffries, T. K. "Degradation of ^{14}C-Triclopyr in Sterile and Non-Sterile Soil", GH-C 960, 1976, unpublished report of DowElanco.

3. Woodburn, K. B.; Cranor, W. "Aerobic Aquatic Metabolism of ^{14}C-Triclopyr", GH-C 1987, 1987, unpublished report of DowElanco.

4. Woodburn, K. B. "The Aquatic Dissipation of Triclopyr in Lake Seminole, Georgia", GH-C 2093, 1988, unpublished report of DowElanco.

5. Woodburn, K. B. Aquatic Dissipation of Triclopyr in Lake Seminole, Georgia. *J. Agric. Food Chem.* **1993**, 41, 2172-2177.

6. Woodburn, K. B. "The Aquatic Dissipation of Triclopyr in Banks Lake, Washington", GH-C 2211, 1989, unpublished report of DowElanco.

7. Woodburn, K. B.; Batzer, F. R.; White, F. H.; Schultz, M. R. "The Aqueous Photolysis of Triclopyr", GH-C 2434, 1990, unpublished report of DowElanco.

8. Woodburn, K. B.; Batzer, F. R.; White, F. H.; Schultz, M. R. The Aqueous Photolysis of Triclopyr. *Environ. Toxicol. Chem.* **1993**, 12, 43-55.

9. Buttler, I. W.; Roberts, D. W.; Siders, L. E.; Gardner, R. C. "Non-Crop Right-of-Way Terrestrial Dissipation of Triclopyr in California", GH-C 3007, 1993, unpublished report of DowElanco.

10. Petty, D. G.; Gardner, R. C. "Right-of-Way Terrestrial Dissipation of Triclopyr in North Carolina", GH-C 3123, 1993, unpublished report of DowElanco.

11. Lickly, T. D.; Murphy, P. G. "The Level and Identification of ^{14}C Residues in Bluegills (*Lepomis macrochirus*) Exposed to 2.5 ppm [^{14}C]Triclopyr", ES-682, 1984, unpublished report of The Dow Chemical Company.

12. Lickly, T. D.; Murphy, P. G. The Amount and Identity of ^{14}C Residues in Bluegills (*Lepomis macrochirus*) Exposed to [^{14}C]Triclopyr. *Environ. Internat.* **1987**, 13, 213-218.

13. Barron, M. G.; Mayes, M. A.; Murphy, P. G.; Nolan, R. J. Pharmacokinetics and Metabolism of Triclopyr Butoxyethyl Ester in Coho Salmon. *Aquat. Toxicol.* **1990**, 16, 19-32.

14. Barron, M. G.; Hansen, S. C.; Ball, T. Pharmacokinetics and Metabolism of Triclopyr in the Crayfish (*Procambarus clarki*). *Drug Metab. Dispos.* **1991**, 19(1), 163-167.

15. Rick, D. L.; Kirk, H. D.; Fontaine, D. D.; Bartels, M. J.; Woodburn, K. B. "The Nature of Triclopyr Residues in the Bluegill, *Lepomis macrochirus* Rafinesque", ES-2761, 1996, unpublished report of The Dow Chemical Company.

16. Olberding, E. L.; Foster, D. R. "Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection", GRM 97.02, 1997, unpublished residue method of DowElanco.

17. Houtman, B. A.; Foster, D. R.; Getsinger, K. D.; Petty, D.G. "Triclopyr Dissipation and the Formation and Decline of its TMP and TCP Metabolites in an Aquatic Environment", GH-C 4499, 1997, unpublished report of DowElanco.

18. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.

APPENDIX A

DowElanco Method GRM 97.02

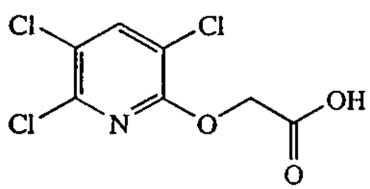
GRM: 97.02
EFFECTIVE: August 19, 1997
SUPERSEDES: ACR 77.4.S1

Determination of Residues of Triclopyr, 3,5,6-Trichloro-2-pyridinol, and 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues by Capillary Gas Chromatography with Mass Selective Detection

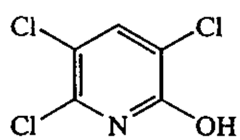
E. L. Olberding and D. R. Foster
Global Environmental Chemistry Laboratory—Indianapolis Lab
DowElanco LLC
Indianapolis, Indiana 46268-1054

A. Scope

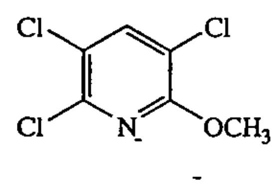
This method is applicable for the quantitative determination of residues of triclopyr (((3,5,6-trichloro-2-pyridinyl)oxy)acetic acid) and its metabolites, 3,5,6-trichloro-2-pyridinol (3,5,6-TCP), and 2-methoxy-3,5,6-trichloropyridine (2-MP) in fish tissues. The method measures total levels of the analytes (both free and conjugated) over the concentration range 0.01-5.0 µg/g with a validated limit of quantitation of 0.01 µg/g.



Triclopyr
CAS No. 55335-06-3



3,5,6-TCP
CAS No. 6515-38-4



2-MP
CAS No. 31557-34-3

B. Principle

Residues of triclopyr, 3,5,6-TCP, and 2-MP are extracted from fish tissues and hydrolyzed using aqueous 0.25 N sodium hydroxide. Following hydrolysis, the sodium hydroxide is acidified and the analytes extracted with ethyl ether. The ethyl ether is passed through an alumina solid-phase extraction (SPE) column which retains the triclopyr and 3,5,6-TCP, while the 2-MP is contained in the ethyl ether eluate. For the determination of 2-MP, the ethyl ether is concentrated and exchanged with 1-chlorobutane. The triclopyr and 3,5,6-TCP are eluted from the alumina column with 0.1 N sodium hydroxide, which is subsequently acidified and purified using a C_{18} SPE procedure. The eluate from the C_{18} SPE is then extracted with 1-chlorobutane. For both sample fractions, the 1-chlorobutane is concentrated to less than 1 mL, and an acetone solution containing fluroxypyr analogs as internal standards is added. The samples are then derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoracetamide (MTBSTFA) to form the *tert*-butyldimethylsilyl (TBDMS) derivatives of triclopyr and 3,5,6-TCP. The samples are then analyzed by capillary gas chromatography with mass selective detection.

C. Safety Precautions

1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non-DowElanco products should be obtained from the container label or from the supplier.
   - Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.

2. Acetone, acetonitrile, 1-chlorobutane, ethyl ether, and methanol are flammable and should be used in well-ventilated areas away from ignition sources.

3. Hydrochloric acid and sodium hydroxide are corrosive and can cause severe burns. It is imperative that proper eye and personal protection equipment be worn when handling these reagents.

D. Equipment (Note L.1.)

1. Balance, analytical, Model AE200, Mettler Instrument Corporation, Hightstown, NJ 08520.

2. Balance, pan, Model BB2440, Mettler Instrument Corporation.

3. Bath, water, catalog number 15-461-20, Fisher Scientific, Pittsburgh, PA 15219.

4. Centrifuge, with rotor to accommodate 40-mL vials, Model Centra-8, International Equipment Company, Needham Heights, MA 02194.

5. Evaporator, N-Evap, Model 111, Organomation Associates, Inc., South Berlin, MA 01549. (Note L.2.)

6. Gas chromatograph, Model 5890A Series II, Hewlett-Packard, Wilmington, DE 19808.

7. Hammer mill, with 3/16-inch screen, Model 2001, AGVISE Laboratories, Inc., Northwood, ND 58267.

8. Injector, automatic, Model 7673, Hewlett-Packard.

9. Mass selective detector, Model 5972A, Hewlett-Packard, Palo Alto, CA 94304.

10. Mass selective detector data system, Model G1034C, Hewlett-Packard.

11. Oven, Model OV-490A-2, Blue M Electric Company, Blue Island, IL 60406.

12. Shaker, variable speed reciprocating with box carrier, Model 6000, Eberbach Corporation, Ann Arbor, MI 48106.

13. Ultrasonic cleaner, Model 1200, Branson Cleaning Equipment Company, Shelton, CT 06484.

14. Vacuum manifold, Model spe-12G, Mallinckrodt Baker, Inc., Phillipsburg, NJ 08865.

15. Vortex mixer, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716.

16. Water purification system, Model Milli-Q UV Plus, Millipore Corporation, Milford, MA 01757.

E. Glassware and Materials (Note L.1.)

1. Column, capillary gas chromatography, Durabond-1701 liquid phase, 10 m x 0.18 mm i.d., 0.4-µm film thickness, catalog number 121-0713, J & W Scientific, Folsom, CA 95630.

2. Column, alumina SPE, catalog number 7214-07, Mallinckrodt Baker, Inc.

3. Column, C_{18} SPE, catalog number 7020-07, Mallinckrodt Baker, Inc.

4. Cylinder, graduated mixing, 1000-mL, catalog number 6224-27, Ace Glass Inc., Vineland, NJ 08360.

5. Filter, charcoal, catalog number 7972, Chrompack, Inc., Raritan, NJ 08869. (Note L.3.)

6. Filter, moisture, catalog number 7971, Chrompack, Inc. (Note L.3.)

7. Filter, oxygen, catalog number 7970, Chrompack, Inc. (Note L.3.)

8. Flask, volumetric, 100-mL, catalog number 161-8987, National Scientific Company, Lawrenceville, GA 30243.

9. Flask, volumetric, 200-mL, catalog number 161-8988, National Scientific Company.

10. Inlet sleeve, double gooseneck splitless, catalog number 5181-3315, Hewlett-Packard.

11. pH indicator sticks, pH range 0-6, catalog number 4391-01, Mallinckrodt Baker, Inc.

12. Pipet, serological, 1.0-mL, catalog number P4760-1C, National Scientific Company.
13. Pipet, volumetric, 1.0-mL, catalog number 261-6011, National Scientific Company.
14. Pipet, volumetric, 2.0-mL, catalog number 261-6012, National Scientific Company.
15. Pipet, volumetric, 2.5-mL, catalog number 261-6084, National Scientific Company.
16. Pipet, volumetric, 3.0-mL, catalog number 261-6013, National Scientific Company.
17. Pipet, volumetric, 4.0-mL, catalog number 261-6014, National Scientific Company.
18. Pipet, volumetric, 5.0-mL, catalog number 261-6015, National Scientific Company.
19. Pipet, volumetric, 10-mL, catalog number 261-6020, National Scientific Company.
20. Pipet, volumetric, 20-mL, catalog number 261-6030, National Scientific Company.
21. Pipet, volumetric, 25-mL, catalog number 261-6035, National Scientific Company.
22. Pipet, volumetric, 50-mL, catalog number 261-6050, National Scientific Company.
23. Syringe, 50-µL, Model 705N, Hamilton Company, Reno, NV 89520.
24. Syringe, 100-µL, Model 710N, Hamilton Company.
25. Syringe, 250-µL, Model 725N, Hamilton Company.
26. Syringe, 500-µL, Model 750N, Hamilton Company.
27. Vial, autosampler, 2-mL, catalog number C4000-1, National Scientific Company.
28. Vial, 12-mL, with PTFE-lined screw cap, catalog number B7800-12, National Scientific Company.
29. Vial, 16-mL, with PTFE-lined screw cap, catalog number B7800-4, National Scientific Company.
30. Vial, 40-mL, with PTFE-lined screw cap, catalog number B7800-6, National Scientific Company.
31. Vial cap, for autosampler vial, catalog number C4000-54B, National Scientific Company.

F. Reagents and Chemicals (Note L.1.)

1. Reagents

a. Acetone, OmniSolv grade, catalog number AX0110-1, EM Science, Gibbstown, NJ 08027.

b. Acetonitrile, OmniSolv grade, catalog number AX0151-1, EM Science.

c. 1-Chlorobutane, OmniSolv grade, catalog number CX0914-1, EM Science.

d Ethyl ether, anhydrous, catalog number EX0190-6, EM Science.

e. Helium, gas, 99.995% purity, Airco, Murray Hill, NJ 07974.

f. Hydrochloric acid, 5.0 N, catalog number LC15360-2, Fisher Scientific.

g. Hydrochloric acid, 1.0 N, catalog number SA48-1, Fisher Scientific.

h. Hydrochloric acid, 0.10 N, catalog number SA54-1, Fisher Scientific.

i. Methanol, OmniSolv grade, catalog number MX-0480-1, EM Science.

j. MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide), catalog number 48920, Pierce Chemical Company, Rockford, IL 61105.

k. Nitrogen, gas, 99.99% purity, Airco.

l. Nitrogen, refrigerated liquid, catalog number LQNI-230, Airco Gas and Gear, Indianapolis, IN 46241.

m. Sodium chloride, ACS reagent grade, catalog number S271-1, Fisher Scientific.

n. Sodium hydroxide, 0.25 N, certified concentration, catalog number SS272-1, Fisher Scientific.

o. Sodium hydroxide, 0.10 N, certified concentration, catalog number SS276-1, Fisher Scientific.

p. Standards

(1) triclopyr (((3,5,6-trichloro-2-pyridinyl)oxy)acetic acid)

(2) 3,5,6-trichloro-2-pyridinol (3,5,6-TCP)

(3) 2-methoxy-3,5,6-trichloropyridine (2-MP)

(4) fluroxypyr (((4-amino-3,5,-dichloro-6-fluoro-2-pyridinyl)oxy)acetic acid)

(5) 4-amino-3,5-dichloro-6-fluoro-2-pyridinol (fluroxypyr-DCP)

(6) 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine (fluroxypyr-MP)

Obtain from Test Substance Coordinator, DowElanco, 9330 Zionsville Road, Building 306/A1, Indianapolis, IN 46268-1054.

2. Prepared Solutions

a. 80% acetonitrile/19% water/1% 1.0 N hydrochloric acid solution (v/v/v).

Pour 800 mL of acetonitrile into a 1000-mL graduated mixing cylinder. Pipet 10.0 mL of 1.0 N hydrochloric acid into the same cylinder; then add approximately 150 mL of water. Swirl the cylinder, and allow to equilibrate to room temperature. Dilute to volume with water.

b. 40% acetonitrile/59% water/1% 1.0 N hydrochloric acid solution (v/v/v).

Pour 400 mL of acetonitrile into a 1000-mL graduated mixing cylinder. Pipet 10.0 mL of 1.0 N hydrochloric acid into the same cylinder; then add approximately 500 mL of water. Swirl the cylinder, and allow to equilibrate to room temperature. Dilute to volume with water.

G. Preparation of Standards

1. Preparation of Spiking Solutions/Calibration Standards

a. Weigh 0.1000 g of triclopyr analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

b. Weigh 0.1000 g of 3,5,6-trichloro-2-pyridinol analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

c. Weigh 0.1000 g of 2-methoxy-3,5,6-trichloropyridine analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

d. Pipet 20.0 mL of each of the stock solutions in Sections G.1.a.-c. into a single 200-mL volumetric flask and adjust to volume with acetone to obtain a solution containing 100.0 µg/mL of each compound

e. Prepare solutions for spiking fish tissues by diluting the solution from Section G.1.d with acetone as follows:

Aliquot of Initial Soln.	Final Soln. Volume	Spiking Soln. Final Conc.	Equivalent Sample Conc.[a]
mL	mL	µg/mL	µg/g
0.031	100	0 031	0.003
0.050	100	0.050	0.005
0.100	100	0.100	0.010
0.250	100	0.250	0.025
0.500	100	0.500	0.050
1.00	100	1.00	0.100
2.50	100	2.50	0.250
5.00	100	5.00	0.500
10.0	100	10.0	1.00
25.0	100	25.0	2.50
50.0	100	50.0	5.00

[a] The equivalent sample concentration is based on fortifying a 1.0-g fish tissue sample with 100 µL of spiking solution.

f. Prepare calibration standards by dispensing 100 µL of the solutions from Section G.1.e. into 12-mL vials containing 0.5 mL of 1-chlorobutane and derivatizing according to the procedure described in Section I.1.ii.-mm. The concentration range of these calibration standards is from 0.005 to 5.0 µg/mL.

Chemical structures of the underivatized and derivatized triclopyr, 3,5,6-TCP, and 2-MP are shown in Figure 1.

2. Preparation of Internal Standard Solution

a. Weigh 0.1000 g of fluroxypyr analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

b. Weigh 0.1000 g of 4-amino-3,5-dichloro-6-fluoro-2-pyridinol analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

c. Weigh 0.1000 g of 4-amino-3,5-dichloro-6-fluoro-2-methoxypyridine analytical standard and quantitatively transfer to a 100-mL volumetric flask. Dilute to volume with acetone to obtain a 1000-µg/mL stock solution.

d. Pipet 20.0 mL of each of the stock solutions in Sections G.2.a.-c. into a single 200-mL volumetric flask and adjust to volume with acetone to obtain a solution containing 100.0 µg/mL of each compound.

e. Pipet 20.0 mL of the solution in Section G.2.d. into a 200-mL volumetric flask and adjust to volume with acetone to obtain a mixture containing 10.0 µg/mL of each compound

Chemical structures of the underivatized and derivatized fluroxypyr, fluroxypyr-DCP, and fluroxypyr-MP are shown in Figure 2.

H. Gas Chromatography/Mass Spectrometry

1. Column

- Install the splitless column inlet sleeve (Section E.10.) and the capillary column (Section E.1.) in the split/splitless injection port of the gas chromatograph following the manufacturer's recommended procedures.

2. Typical Operating Conditions (Note L.4.)

Instrumentation:	Hewlett-Packard Model 5890A Gas Chromatograph Hewlett-Packard Model 7673 Automatic Sampler Hewlett-Packard Model 5972A Mass Selective Detector Hewlett-Packard Model G1034C Data System Software
Column:	J & W Scientific fused silica capillary Durabond-1701 liquid phase 10 m x 0.18 mm i.d. 0.4-µm film thickness
Temperatures:	
Column	70 °C for 1.0 min 70 °C to 255 °C at 10 °C/min 255 °C to 280 °C at 20 °C/min 280 °C for 4.75 min
Injector	280 °C
Interface	280 °C
Carrier Gas:	helium
Constant Flow	on
Vacuum Compensation	on
Head Pressure	25 kPa
Linear Velocity	approximately 45 cm/s

Injection Mode:	splitless
Purge Delay	0.9 min
Splitter Flow	50 mL/min
Septum Purge	1.0 mL/min
Injection Volume:	1 μL
Detector:	electron impact selected ion monitoring
Calibration Program	maximum sensitivity autotune (Note L.5.)
Electron Multiplier	2050 volts (≈280 volts above autotune)
Ions Monitored: (Note L.6.)	
Triclopyr-TBDMS	*m/z* 314 (quantitation) *m/z* 254, 256, 312 (confirmation) (Section K.2.)
3,5,6-TCP-TBDMS	*m/z* 254 (quantitation) *m/z* 256 (confirmation)
2-MP	*m/z* 213 (quantitation) *m/z* 182, 210, 211, 212 (confirmation) (Section K.2.)
Fluroxypyr-TBDMS	*m/z* 311 (internal standard for triclopyr-TBDMS)
Fluroxypyr-DCP-TBDMS	*m/z* 253 (internal standard for 3,5,6-TCP-TBDMS)
Fluroxypyr-MP	*m/z* 210 (internal standard for 2-MP)
Dwell Time	75 ms

Mass spectra of the above triclopyr and fluroxypyr compounds are shown in Figures 3-5, respectively.

3. Calibration Curves

Typical calibration curves for the determination of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues are shown in Figures 6-8, respectively.

4. Typical Chromatograms

Typical chromatograms of a standard, control sample, and a 0.01-μg/g recovery sample for the determination of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues are illustrated in Figures 9-17, respectively. Crayfish inedible tissue was chosen since it represents the "worst case" situation for sample preparation and analysis.

I. Determination of Recovery of Triclopyr and Metabolites from Fish Tissues

1. Preparation of Recovery Samples

a. Prepare the tissue sample for analysis by freezing with liquid nitrogen and then grinding the sample using a hammer mill with a 3/16-inch screen.

b. Accurately weigh 1.0-g portions of the prepared fish tissue sample into a series of 40-mL vials.

c. For preparing fortified samples, use some of the samples as controls and fortify the remaining samples by adding 100-µL aliquots of the appropriate spiking solutions (Section G.1.e.) to obtain concentrations ranging from 0.01 to 5.0 µg/g. A reagent blank, containing no fish tissue, should be carried through the method with the samples.

d. Add 10.0 mL of the 0.25 N sodium hydroxide extraction solution to the sample vial.

e. Cap the sample vial with a PTFE-lined cap and vortex the sample for 5-10 seconds.

f. Hydrolyze the sample by heating in a water bath for 2 hours at 80 °C.

g. After hydrolysis, allow the sample to cool to room temperature.

h. Using a serological pipet, add 0.75 mL of 5.0 N hydrochloric acid to the sample vial and swirl gently to mix. Measure the pH to insure that the pH<1. If necessary, adjust the pH by the dropwise addition of 5.0 N hydrochloric acid. (Note L.7.)

i. Add 5 g of sodium chloride (enough to saturate the solution) and 10 mL of ethyl ether to the sample vial.

j. Cap the sample vial with a PTFE-lined cap, and shake the sample for a minimum of 1 hour on a reciprocating shaker at approximately 250 excursions/minute.

k. Centrifuge the sample vial for 5 minutes at approximately 2000 rpm.

l. Transfer the ethyl ether (top) layer into a clean 16-mL vial.

m. Add an additional 5 mL of ethyl ether to the sample vial. Cap the vial, and shake the sample for an additional hour on a reciprocating shaker at approximately 250 excursions/minute.

n. Centrifuge the sample vial for 5 minutes at approximately 2000 rpm.

o. Transfer the ethyl ether layer from Step I.1.n. into the same vial containing the ethyl ether from Step I.1.l.

p. Repeat Steps I.1.m.-o. one additional time. After transferring the ethyl ether layer into the 16-mL vial from the first extraction, discard the 40-mL vial containing the aqueous portion of the sample extract.

q. Concentrate the solution from Step I.1.p. to 5 ± 0.5 mL using an N-Evap evaporator. (Note L.8.)

r. Purify the sample using the following alumina SPE procedure (Sections K.4.a. and K 4.b.):

(1) Place an alumina SPE column on the vacuum manifold.

(2) Rinse the SPE column with 5 mL of methanol. (Do not allow the column bed to dry.)

(3) Condition the SPE column with 5 mL of ethyl ether. (Do not allow the column bed to dry.)

(4) Place a 12-mL vial in the vacuum manifold box to collect the eluate in the following step.

(5) Transfer the sample solution from Step I.1.q. to the SPE column and allow the sample to flow through the column by gravity alone, collecting the eluate in the 12-mL vial. (Do not allow the column bed to dry.)

(6) Rinse the sample vial with 2 mL of ethyl ether. When the sample solution in Step I.1.r.(5) is within 2 mm of the top of the column bed, transfer the rinse to the SPE column. Allow the solvent to flow through the column by gravity alone, collecting the eluate in the same 12-mL vial.

(7) Remove the 12-mL vial from the vacuum manifold box and set it aside. (This extract, containing the 2-methoxy-3,5,6-trichloropyridine, will be further treated as described in Steps I.1.s.-x.)

(8) Rinse the sample vial with 5 mL of acetone and transfer the rinse to the SPE column. Allow the solvent to flow through the column by gravity alone, and discard the eluate.

(9) Dry the SPE column under vacuum for 5 minutes.

(10) Place a 40-mL vial in the vacuum manifold box to collect the eluate in the following step.

(11) Elute the triclopyr and 3,5,6-trichloro-2-pyridinol from the alumina SPE column by passing 25 mL of 0.1 N sodium hydroxide solution through the column at a flow rate of 1-2 mL/min, collecting the eluate in the 40-mL vial. (This extract, containing the triclopyr and 3,5,6-trichloro-2-pyridinol, will be further treated as described in Steps I 1.y.-nn.)

2-Methoxy-3,5,6-trichloropyridine

s. Concentrate the solution from Step I.1.r.(7) to approximately 2 mL using an N-Evap evaporator. (Note L.8.)

t. Add 2.0 mL of 1-chlorobutane and continue concentrating the solution from Step I.1.s. to less than 0.8 mL (but not to dryness) using an N-Evap evaporator. (Notes L.2. and L.8.)

u. Add 100 µL of the internal standard solution (Section G.2.e) and 100 µL of MTBSTFA derivatizing reagent to the sample vial.

v. Adjust the volume in the sample vial to 1.0 mL with 1-chlorobutane and firmly seal with a PTFE-lined cap (Note L.8.). Vortex the sample for 5-10 seconds, and then sonicate the sample for 5-10 seconds.

w. Transfer the solution to a 2-mL autosampler vial and seal with a cap.

x. Analyze the calibration standards (Section G.1.f.) and samples by capillary gas chromatography/mass spectrometry as described in Section H.2. Determine the suitability of the chromatographic system using the following performance criteria:

(1) Standard curve linearity: Determine that the correlation coefficient equals or exceeds 0.995 for the least squares equation which describes the detector response as a function of standard curve concentration. If power regression is used, the power exponent should be between 0.90-1.10.

(2) Peak resolution: Visually determine that sufficient resolution has been achieved for the analyte and internal standard relative to background interferences.

(3) Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 15-17 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for the 2-methoxy-3,5,6-trichloropyridine in the 0.010-µg/mL calibration standard.

Triclopyr and 3,5,6-Trichloro-2-pyridinol

y. Using a serological pipet, add 1.1 mL of 5.0 N hydrochloric acid to the sample vial from Step I.1.r.(11), cap the vial, and vortex the sample for 5-10 seconds.

z. Purify the sample using the following C_{18} SPE (Section K.4.c.):

(1) Place a C_{18} SPE column on the vacuum manifold.

(2) Rinse the SPE column with 5 mL of acetonitrile. (Do not allow the column bed to dry.)

(3) Condition the SPE column with 5 mL of 0.1 N hydrochloric acid. (Do not allow the column bed to dry.)

(4) Transfer the sample solution from Step I.1.y. to the SPE column, and slowly pull the sample through the column at a flow rate of 1-2 mL/min with the aid of vacuum. Discard the eluate without allowing the column bed to dry.

(5) Rinse the sample vial with 4.0 mL of a 40% acetonitrile/59% water/1% 1.0 N hydrochloric acid solution. When the sample solution in Step I.1.z.(4) is within 2 mm of the top of the column bed, transfer the rinse to the SPE column. With the aid of vacuum, slowly pull the rinse solution through the column, and discard the eluate.

(6) Dry the SPE column under vacuum for 1 minute.

(7) Place a 40-mL vial in the vacuum manifold box to collect the eluate in the following step.

(8) Elute the triclopyr and 3,5,6-trichloro-2-pyridinol from the C_{18} SPE column by passing 3.0 mL of an 80% acetonitrile/19% water/1% 1.0 N hydrochloric acid solution through the column, collecting the eluate in the 40-mL vial.

aa. Add 10 mL of 0.1 N hydrochloric acid, 5 g of sodium chloride (enough to saturate the solution), and 5 mL of 1-chlorobutane to the sample vial.

bb. Cap the sample vial with a PTFE-lined cap, and shake the sample for a minimum of 10 minutes on a reciprocating shaker at approximately 180 excursions/minute.

cc. Centrifuge the sample vial for 5 minutes at approximately 2000 rpm.

dd Transfer the 1-chlorobutane layer into a clean 12-mL vial. (Note L.9.)

ee. Add an additional 5 mL of 1-chlorobutane to the sample vial. Cap the vial, and shake the sample for an additional 20 minutes on a reciprocating shaker at approximately 180 excursions/minute.

ff. Centrifuge the sample vial for 5 minutes at approximately 2000 rpm.

gg. Transfer the 1-chlorobutane layer from Step I.1.ff. into the same vial containing the 1-chlorobutane from Step I.1.dd. (Note L.9.)

hh. Concentrate the solution from Step I.1.gg. to less than 0.8 mL (but not to dryness) using an N-Evap evaporator. (Notes L.2. and L.8.)

ii Add 100 µL of the internal standard solution (Section G.2.e) and 100 µL of MTBSTFA derivatizing reagent to the sample vial.

jj. Adjust the volume in the sample vial to 1.0 mL with 1-chlorobutane and firmly seal with a PTFE-lined cap (Note L.8.). Vortex the sample for 5-10 seconds, and then sonicate the sample for 5-10 seconds.

kk. Place the sample vial in an oven at 60 °C and allow the mixture to react for 60 minutes.

ll. Remove the sample vial from the oven and allow the reaction mixture to cool to room temperature.

mm. Transfer the solution to a 2-mL autosampler vial and seal with a cap.

nn. Analyze the calibration standards (Section G.1.f.) and samples by capillary gas chromatography/mass spectrometry as described in Section H.2. Determine the suitability of the chromatographic system using the following performance criteria:

(1) Standard curve linearity: Determine that the correlation coefficients equal or exceed 0.995 for the least squares equations which describes the detector response as a function of standard curve concentration. If power regression is used, the power exponents should be between 0.90-1.10.

(2) Peak resolution: Visually determine that sufficient resolution has been achieved for the analytes and internal standards relative to background interferences.

(3) Appearance of chromatograms: Visually determine that the chromatograms resemble those shown in Figures 9-14 with respect to peak response, baseline noise, and background interference. Visually determine that a minimum signal-to-noise ratio of 10:1 has been attained for each analyte in the 0.010-µg/mL calibration standard.

2. Calculation of Percent Recovery

a. Inject the series of calibration standards described in Section G.1.f. and determine the peak areas for the analytes and internal standards as indicated below.

Triclopyr-TBDMS	*m/z* 314 (quantitation), *m/z* 312 (confirmation)
3,5,6-TCP-TBDMS	*m/z* 254 (quantitation), *m/z* 256 (confirmation)
2-MP	*m/z* 213 (quantitation), *m/z* 211 (confirmation)
Fluroxypyr-TBDMS	*m/z* 311 (internal standard for triclopyr-TBDMS)
Fluroxypyr-DCP-TBDMS	*m/z* 253 (internal standard for 3,5,6-TCP-TBDMS)
Fluroxypyr MP	*m/z* 210 (internal standard for 2-MP)

b. For each standard, calculate each analyte's confirmation ratio. Use the average confirmation ratio for each analyte to confirm the presence of the analyte in the fish tissue samples.

For example, using the data for triclopyr from Figure 9:

$$\text{Confirmation Ratio} = \frac{\text{peak area of confirmation ion}}{\text{peak area of quantitation ion}}$$

$$\text{Confirmation Ratio} = \frac{\text{peak area at } m/z\ 312}{\text{peak area at } m/z\ 314}$$

$$\text{Confirmation Ratio} = \frac{16597}{16562}$$

$$\text{Confirmation Ratio} = 1.0021$$

Confirmation of the presence of the analyte is indicated when the confirmation ratio for the sample is within the range of ±20% of the average found for the standards.

c. For each standard, calculate each analyte's quantitation ratio.

For example, using the data for triclopyr from Figure 9:

$$\text{Quantitation Ratio} = \frac{\text{peak area of quantitation ion}}{\text{peak area of internal standard ion}}$$

$$\text{Quantitation Ratio} = \frac{\text{peak area at } m/z\ 314}{\text{peak area at } m/z\ 311}$$

$$\text{Quantitation Ratio} = \frac{16562}{1723923}$$

$$\text{Quantitation Ratio} = 0.00961$$

d. Prepare a standard curve for each analyte by plotting the equivalent analyte concentration on the abscissa (x-axis) and the respective quantitation ratio on the ordinate (y-axis) as shown in Figures 6-8. Using regression analysis, determine the equation for the curve with respect to the abscissa.

For example, using power regression (1) with the triclopyr data from Figure 6:

$$Y = \text{constant} \times X^{(\text{exponent})}$$

$$X = \left(\frac{Y}{\text{constant}}\right)^{1/\text{exponent}}$$

$$\underset{(\mu g/g)}{\text{Triclopyr Conc.}} = \left(\frac{\text{triclopyr quantitation ratio}}{\text{constant}}\right)^{1/\text{exponent}}$$

$$\underset{(\mu g/g)}{\text{Triclopyr Conc.}} = \left(\frac{\text{triclopyr quantitation ratio}}{0.8925}\right)^{1/1.0139}$$

e. Determine the gross concentration in each recovery sample by substituting the quantitation ratio obtained into the above equation and solving for the concentration.

For example, using the triclopyr data from Figure 11:

$$\underset{(\text{gross } \mu g/g)}{\text{Triclopyr Conc.}} = \left(\frac{\text{triclopyr quantitation ratio}}{0.8925}\right)^{1/1.0139}$$

$$\underset{(\text{gross } \mu g/g)}{\text{Triclopyr Conc.}} = \left(\frac{0.0085}{0.8925}\right)^{1/1.0139}$$

$$\text{Triclopyr Conc.} = 0.0102\ \mu g/g$$

f. Determine the net concentration in each recovery sample by subtracting the triclopyr concentration in the control sample from that of the gross triclopyr concentration in the recovery sample.

For example, using the triclopyr data from Figures 10 and 11:

$$\underset{(\text{net } \mu g/g)}{\text{Triclopyr Conc.}} = \underset{(\text{gross } \mu g/g)}{\text{Triclopyr Conc.}} - \underset{(\text{control } \mu g/g)}{\text{Triclopyr Conc.}}$$

$$\underset{(\text{net } \mu g/g)}{\text{Triclopyr Conc.}} = 0.0102\ \mu g/g - 0.0000\ \mu g/g$$

$$\underset{(\text{net})}{\text{Triclopyr Conc.}} = 0.0102\ \mu g/g$$

g. Determine the percent recovery by dividing the net concentration of each recovery sample by the theoretical concentration added.

$$\text{Recovery} = \frac{\text{Concentration Found}}{\text{Concentration Added}} \times 100\%$$

$$\text{Recovery} = \frac{0.0102\ \mu g/g}{0.0100\ \mu g/g} \times 100\%$$

$$\text{Recovery} = 102\%$$

J. Determination of Triclopyr and Metabolites in Fish Tissues

1. Prepare reagent blank, control, recovery, and treated samples as described in Section I.1.

2. Prepare a standard calibration curve for triclopyr, 3,5,6-TCP, and 2-MP and determine the percent recovery for each analyte as described in Section I.2.

3. Determine the gross concentration of each analyte in each treated sample by substituting the quantitation ratio obtained into the equation for the standard calibration curve, and calculating the uncorrected residue result as described in Section I.2.e.

4. For those analyses that require correction for method recovery, use the average recovery of all the recovery samples from a given sample set to correct for method efficiency.

   For example, using the triclopyr data from Figure 11 and Table I for the samples analyzed on 3-Jul-1997:

   a. Determine the gross analyte concentrations in the fish tissue sample as described in Section I.2.e.

   b. Determine the corrected analyte concentration in the fish tissue sample as follows:

$$\underset{(\text{corrected } \mu g/g)}{\text{Triclopyr Conc.}} = \underset{(\text{gross } \mu g/g)}{\text{Triclopyr Conc.}} \times \left(\frac{100}{\text{Average Percent Recovery}}\right)$$

$$\underset{(\text{corrected } \mu g/g)}{\text{Triclopyr Conc.}} = 0.0102\ \mu g/g \times \frac{100}{92}$$

$$\underset{(\text{corrected})}{\text{Triclopyr Conc.}} = 0.0111\ \mu g/g$$

K. Results and Discussion

1. Method Validation

a. Recovery Levels and Precision

A method validation study was conducted to determine the recovery levels and the precision of the method for the determination of triclopyr, 3,5,6-TCP, and 2-MP in fish tissues. The results are summarized in Tables I-III.

Recovery values of triclopyr from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 µg/g averaged 93% with one standard deviation equal to 6% (Table I). Recovery values of 3,5,6-TCP from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 µg/g averaged 92% with one standard deviation equal to 9% (Table II). Recovery values of 2-MP from samples of fish tissues fortified over the concentration range of 0.01 to 5.0 µg/g averaged 92% with one standard deviation equal to 8% (Table III).

b. Standard Curve Linearity

For the power least squares regression equations describing the detector response as a function of the standard calibration curve concentrations, correlation coefficients (r^2) were greater than 0.999 for all three analytes, while the power exponents were between 0.97 and 1.01.

c. Calculated Limits of Quantitation and Detection

Following established guidelines (2), the limits of quantitation (LOQ) and detection (LOD) were calculated using the standard deviation from the 0.01-µg/g recovery results. The LOQ was calculated as ten times the standard deviation (10*s*), and the LOD was calculated as three times the standard deviation (3*s*) of the results of the analysis of 12 samples. The results are summarized in Tables I-III.

For all three analytes, the calculated LOQ ranged from 0.007 to 0.009 µg/g, which is lower than the targeted method LOQ of 0.01 µg/g. In a similar fashion, for all three analytes the calculated LOD ranged from 0.002 to 0.003 µg/g. However, since the lowest level of fortification for recovery samples was 0 003 µg/g, the method LOD is considered to be 0.003 µg/g.

2. Confirmation of Residue Identity

Confirmation of the presence of residues is described in Section I.2.b. For the three analytes, confirmation is by comparison of the retention time (gas chromatography) as well as the peak area ratios resulting from selected ion monitoring (mass spectrometry). Confirmation of the presence of the analytes is indicated when the confirmation ratio for the sample is in the range of ±20% of the average found for the standards. If additional confirmation is required beyond that discussed in this method, the mass spectra of triclopyr-TBDMS and 2-MP contain additional ions (Section H.2.) that may be used for confirmation.

3. Assay Time

A typical analytical run would consist of a minimum of four standards encompassing the expected range of sample concentrations, a reagent blank, a control (a non-fortified sample), a minimum of two fortified controls (one of which must be at the LOQ), and 15 samples. This typical analytical run could be prepared in approximately 24 hours (three working days).

There are several acceptable "stopping points" in the method, where sample preparation (Section I) may be suspended, upon completion of a step, without deleterious effects on the sample analysis. These are indicated below:

a. Step I.1.p. In preparing large sets of samples, this is a convenient stopping point at the end of the first 8-hour day.
b. Step I.1.q.
c. Step I.1.r.(7).
d. Step I.1 s.
e. Step I.1.w. In preparing large sets of samples, this is a convenient stopping point at the end of the second 8-hour day.
f. Step I.1.z.(8).
g. Step I.1.gg
h. Step I.1.mm

If the samples are to be stored overnight, the vials should be capped with PTFE-lined caps.

4. Standardization of SPE Elution Profiles

Variation in the alumina and C_{18} SPE columns may influence the elution profiles of triclopyr, 3,5,6-TCP, and 2-MP. It is necessary to obtain an elution profile for each lot of SPE columns used to ensure optimum recovery and clean-up efficiency. The following procedures can be used:

a. Alumina SPE Profile for 2-MP

(1) To a 12-mL vial containing 5 mL of ethyl ether, add 50 µL of the 100-µg/mL spiking solution (Section G.1.d.).

(2) Place an alumina SPE column on the vacuum manifold.

(3) Rinse the SPE column with 5 mL of methanol. (Do not allow the column bed to dry.)

(4) Condition the SPE column with 5 mL of ethyl ether. (Do not allow the column bed to dry.)

(5) Transfer the sample solution from Step K.4.a.(1) to the SPE column and allow the sample to flow through the column by gravity alone, collecting 1-mL aliquots in 12-mL vials. (Do not allow the column bed to dry.)

(6) Rinse the sample vial with 5 mL of ethyl ether, and when the sample solution in Step K.4.a.(5) is within 2 mm of the top of the column bed, transfer the rinse to the SPE column. Allow the solvent to flow through the column by gravity alone, and continue to collect 1-mL aliquots in 12-mL vials.

(7) For each fraction collected, add 2.0 mL of 1-chlorobutane to the sample vial.

(8) Concentrate the solutions to less than 0.8 mL (but not to dryness) using an N-Evap evaporator. (Notes L.2. and L.8.)

(9) Proceed as described in Section I.1.u. through I.1.x.

(10) Calculate the percent recovery for 2-MP as described in Section I.2.

A typical elution profile is illustrated in Figure 18. If the elution profile differs from that shown, adjust the volume of ethyl ether to be collected in Step I.1.r.(6).

b. Alumina SPE Profiles for Triclopyr and 3,5,6-TCP

(1) To a 12-mL vial containing 5 mL of ethyl ether, add 10 µL of the 100-µg/mL spiking solution (Section G.1.d.).

(2) Place an alumina SPE column on the vacuum manifold.

(3) Rinse the SPE column with 5 mL of methanol. (Do not allow the column bed to dry.)

(4) Condition the SPE column with 5 mL of ethyl ether. (Do not allow the column bed to dry.)

(5) Transfer the sample solution from Step K.4.b.(1) to the SPE column and allow the sample to flow through the column by gravity alone, discarding the eluate. (Do not allow the column bed to dry.)

(6) Rinse the sample vial with 2 mL of ethyl ether, and when the sample solution in Step K.4.b.(5) is within 2 mm of the top of the column bed, transfer the rinse to the SPE column. Allow the solvent to flow through the column by gravity alone, discarding the eluate.

(7) Rinse the sample vial with 5 mL of acetone and transfer the rinse to the SPE column. Allow the solvent to flow through the column by gravity alone, discarding the eluate.

(8) Dry the SPE column under vacuum for 5 minutes.

(9) Elute the triclopyr and 3,5,6-TCP from the alumina SPE column by passing 50 mL of 0.1 N sodium hydroxide solution through the column at a flow rate of 1-2 mL/min, collecting 5.0-mL aliquots in 40-mL vials.

(10) For each fraction collected, proceed as described in Section I.1.aa. through I.1.nn.

(11) Calculate the percent recovery for triclopyr and 3,5,6-TCP as described in Section I.2.

Typical elution profiles are illustrated in Figure 18. If the elution profiles differ from those shown, adjust the volume of 0 1 N sodium hydroxide to be collected in Step I.1.r.(11).

c C_{18} SPE Profiles for Triclopyr and 3,5,6-TCP

(1) To two 40-mL vials containing 25 mL of 0.1 N hydrochloric acid, add 10 µL of the 100-µg/mL spiking solution (Section G.1.d.).

(2) Place two C_{18} SPE columns on the vacuum manifold.

(3) Rinse each SPE column with 5 mL of acetonitrile. (Do not allow the column beds to dry.)

(4) Condition each SPE column with 5 mL of 0.1 N hydrochloric acid. (Do not allow the column beds to dry)

(5) Transfer the sample solutions from Step K.4.c.(1) to the SPE columns, and slowly pull the samples through the columns at a flow rate of 1-2 mL/min with the aid of vacuum. Discard the eluates without allowing the column beds to dry.

(6) Rinse the sample vials with 2 mL of 0.1 N hydrochloric acid, and transfer the rinses to the SPE columns. Slowly pull the rinse solutions through the columns at a flow rate of 1-2 mL/min with the aid of vacuum, and discard the eluates.

(7) Dry each SPE column under vacuum for 1 minute.

(8) Elute the triclopyr and 3,5,6-TCP from one of the C_{18} SPE columns with 10 mL of the 40% acetonitrile/59% water/1% 1.0 N hydrochloric acid solution, collecting 1.0-mL aliquots in 40-mL vials.

Elute the triclopyr and 3,5,6-TCP from the other C_{18} SPE column with 10 mL of the 80% acetonitrile/19% water/1% 1.0 N hydrochloric acid solution, collecting 1.0-mL aliquots in 40-mL vials.

(9) For each fraction collected, proceed as described in Section I.1.aa. through I.1.nn.

(10) Calculate the percent recovery for triclopyr and 3,5,6-TCP as described in Section I.2.

Typical elution profiles are illustrated in Figure 19. If the elution profiles differ from that shown, adjust the volume of the 40% acetonitrile/59% water/1% 1.0 N hydrochloric acid solution to be discarded in Step I.1.z.(5) or the volume of the 80% acetonitrile/19% water/1% 1.0 N hydrochloric acid solution to be collected in Step I.1.z.(8)

L. Notes

1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory supplies are assumed to be readily available and are, therefore, not listed

2. The N-Evap evaporator should be set at a water bath temperature of 30 °C and a nitrogen flow rate of approximately 200 mL/min. At elevated water bath temperatures and/or flow rates, the 3,5,6-TCP and 2-MP may volatilize, thereby reducing recoveries.

3. The filters are used in the carrier gas supply lines to purify the helium entering the gas chromatograph.

4. While the sample preparation of the fish tissues for the determination of triclopyr and 3,5,6-TCP differs from that for the determination of 2-MP, the gas chromatographic/mass spectrometric conditions are the same.

5 Several tuning, or calibration, options are available for the Model 597X series of MSDs. The "Maximum Sensitivity Autotune" and "Mid-Mass Autotune" calibration features were found to consistently yield approximately 5-10 times the sensitivity compared to that of the "Standard Autotune" calibration. In addition, either of these calibrations should be followed with a "Usertune" calibration at *m/z* 131, 219, and 264.

6. The ions monitored for the analytes and their respective internal standards are grouped by retention time so that only those ions representative of a given compound are monitored at that compound's retention time

7. Fish tissues containing bony material or shells will react with the 5.0 N hydrochloric acid and may necessitate further addition of 5.0 N hydrochloric acid to maintain a pH<1.

8. The measurement of a non-critical volume (one that does not affect the calculations) can be most easily accomplished by comparing the height (volume) in the sample vial with another vial containing the desired measured volume.

9. In transferring the 1-chlorobutane layer, it is important not to remove any water. Contaminating the 1-chlorobutane with water will have deleterious effects on the derivatization and subsequent GC/MSD analysis

M. References

1. Freund, J. E.; Williams, F. J. *Dictionary/Outline of Basic Statistics*; Dover. New York, 1991; p 170.

2. Keith, L. H.; Crummett, W ; Deegan, J , Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. *Anal. Chem.* **1983**, *55*, 2210-2218.

Table I. Recovery of Triclopyr from Fish Tissues

Sample Number	Date of Analysis	Triclopyr, µg/g Added	Triclopyr, µg/g Found	Percent Recovery	Statistical Calculations
SN17993002[a]	26-Jun-1997	0.000	0.000	--	
SN17993003[b]	3-Jul-1997	0.000	0.000	--	
SN18003302[c]	26-Jun-1997	0.000	0.000	--	
SN18003303[d]	3-Jul-1997	0.000	0 000	--	
SN20178201[e]	26-Jun-1997	0.000	0.000	--	
SN20178202[f]	3-Jul-1997	0.000	0 000	--	
SN17993002	26-Jun-1997	0.003	< 0 010	NA[g]	
SN17993003	3-Jul-1997	0.003	< 0 010	NA	
SN18003302	26-Jun-1997	0.003	< 0.010	NA	
SN18003303	3-Jul-1997	0.003	< 0 010	NA	
SN20178201	26-Jun-1997	0.003	< 0.010	NA	
SN20178202	3-Jul-1997	0.003	< 0.010	NA	
SN17993002	26-Jun-1997	0.010	0.0097	97	
SN17993002	26-Jun-1997	0.010	0.0094	94	
SN17993003	3-Jul-1997	0.010	0.0077	77	
SN17993003	3-Jul-1997	0.010	0.0092	92	
SN18003302	26-Jun-1997	0.010	0.0095	95	
SN18003302	26-Jun-1997	0.010	0.0097	97	
SN18003303	3-Jul-1997	0.010	0.0090	90	
SN18003303	3-Jul-1997	0.010	0.0095	95	$\bar{x}$ = 0 0095
SN20178201	26-Jun-1997	0.010	0.0104	104	s = 0.0007
SN20178201	26-Jun-1997	0.010	0.0095	95	$(3s)$[h] = 0.0021
SN20178202	3-Jul-1997	0.010	0.0102	102	$(10s)$[i] = 0.0071
SN20178202	3-Jul-1997	0.010	0 0103	103	RSD = 7.5%
SN17993002	26-Jun-1997	0.050	0 0456	91	
SN17993003	3-Jul-1997	0.050	0.0465	93	
SN18003302	26-Jun-1997	0.050	0.0518	104	
SN18003303	3-Jul-1997	0.050	0.0473	95	$\bar{x}$ = 0 0478
SN20178201	26-Jun-1997	0.050	0.0466	93	s = 0.0022
SN20178202	3-Jul-1997	0.050	0.0488	98	RSD = 4.7%
SN17993002	26-Jun-1997	0.500	0.483	97	
SN17993003	3-Jul-1997	0.500	0 450	90	
SN18003302	26-Jun-1997	0.500	0 461	92	
SN18003303	3-Jul-1997	0.500	0.431	86	$\bar{x}$ = 0 451
SN20178201	26-Jun-1997	0.500	0.436	87	s = 0.019
SN20178202	3-Jul-1997	0.500	0.445	89	RSD = 4.2%

Table I. (Cont.) Recovery of Triclopyr from Fish Tissues

Sample Number	Date of Analysis	Triclopyr, µg/g		Percent Recovery	Statistical Calculations
		Added	Found		
SN17993002	26-Jun-1997	5.00	4.562	91	
SN17993003	3-Jul-1997	5.00	4.614	92	
SN18003302	26-Jun-1997	5.00	4.266	85	
SN18003303	3-Jul-1997	5.00	4.539	91	$\bar{x}$ = 4 489
SN20178201	26-Jun-1997	5.00	4.318	86	s = 0.157
SN20178202	3-Jul-1997	5.00	4.633	93	RSD = 3.5%
			$\bar{x}$ =	93	
			s =	6	
			n =	30	

[a] SN17993002—Bluegill edible tissue.
[b] SN17993003—Bluegill inedible tissue.
[c] SN18003302—Catfish edible tissue.
[d] SN18003303—Catfish inedible tissue.
[e] SN20178201—Crayfish edible tissue.
[f] SN20178202—Crayfish inedible tissue.
[g] NA = not applicable. The residue was below the 0.010-µg/g limit of quantitation.
[h] Calculated limit of detection.
[i] Calculated limit of quantitation.

Table II. Recovery of 3,5,6-Trichloro-2-pyridinol from Fish Tissues

Sample Number	Date of Analysis	3,5,6-TCP, µg/g Added	3,5,6-TCP, µg/g Found	Percent Recovery	Statistical Calculations
SN17993002[a]	26-Jun-1997	0.000	0.000	--	
SN17993003[b]	3-Jul-1997	0.000	0.000	--	
SN18003302[c]	26-Jun-1997	0.000	0.000	--	
SN18003303[d]	3-Jul-1997	0.000	0.000	--	
SN20178201[e]	26-Jun-1997	0.000	0.000	--	
SN20178202[f]	3-Jul-1997	0.000	0.000	--	
SN17993002	26-Jun-1997	0.003	< 0.010	NA[g]	
SN17993003	3-Jul-1997	0.003	< 0.010	NA	
SN18003302	26-Jun-1997	0.003	< 0.010	NA	
SN18003303	3-Jul-1997	0.003	< 0.010	NA	
SN20178201	26-Jun-1997	0.003	< 0.010	NA	
SN20178202	3-Jul-1997	0.003	< 0.010	NA	
SN17993002	26-Jun-1997	0.010	0.0092	92	
SN17993002	26-Jun-1997	0.010	0.0093	93	
SN17993003	3-Jul-1997	0.010	0.0115	115	
SN17993003	3-Jul-1997	0.010	0.0115	115	
SN18003302	26-Jun-1997	0.010	0.0094	94	
SN18003302	26-Jun-1997	0.010	0.0092	92	
SN18003303	3-Jul-1997	0.010	0.0100	100	
SN18003303	3-Jul-1997	0.010	0.0102	102	$\bar{x}$ = 0.0099
SN20178201	26-Jun-1997	0.010	0.0104	104	s = 0.0009
SN20178201	26-Jun-1997	0.010	0.0102	102	$(3s)$[h] = 0.0026
SN20178202	3-Jul-1997	0.010	0.0091	91	$(10s)$[i] = 0.0086
SN20178202	3-Jul-1997	0.010	0 0092	92	RSD = 8.6%
SN17993002	26-Jun-1997	0.050	0 0445	89	
SN17993003	3-Jul-1997	0.050	0 0463	93	
SN18003302	26-Jun-1997	0.050	0.0431	86	
SN18003303	3-Jul-1997	0.050	0.0451	90	$\bar{x}$ = 0.0450
SN20178201	26-Jun-1997	0.050	0.0502	100	s = 0.0031
SN20178202	3-Jul-1997	0.050	0.0409	82	RSD = 7.0%
SN17993002	26-Jun-1997	0.500	0.406	81	
SN17993003	3-Jul-1997	0 500	0 422	84	
SN18003302	26-Jun-1997	0.500	0.379	76	
SN18003303	3-Jul-1997	0.500	0.409	82	$\bar{x}$ = 0.412
SN20178201	26-Jun-1997	0.500	0 473	95	s = 0.034
SN20178202	3-Jul-1997	0.500	0.383	77	RSD = 8.3%

Table II. (Cont.) Recovery of 3,5,6-Trichloro-2-pyridinol from Fish Tissues

Sample Number	Date of Analysis	3,5,6-TCP, µg/g Added	3,5,6-TCP, µg/g Found	Percent Recovery	Statistical Calculations
SN17993002	26-Jun-1997	5.00	4.359	87	
SN17993003	3-Jul-1997	5.00	4.585	92	
SN18003302	26-Jun-1997	5.00	4.248	85	
SN18003303	3-Jul-1997	5.00	4.581	92	$\bar{x}$ = 4.484
SN20178201	26-Jun-1997	5.00	4.911	98	s = 0.262
SN20178202	3-Jul-1997	5.00	4.220	84	RSD = 5 8%
				$\bar{x}$ = 92	
				s = 9	
				n = 30	

[a] SN17993002—Bluegill edible tissue.
[b] SN17993003—Bluegill inedible tissue
[c] SN18003302—Catfish edible tissue.
[d] SN18003303—Catfish inedible tissue.
[e] SN20178201—Crayfish edible tissue.
[f] SN20178202—Crayfish inedible tissue.
[g] NA = not applicable. The residue was below the 0 010-µg/g limit of quantitation.
[h] Calculated limit of detection.
[i] Calculated limit of quantitation

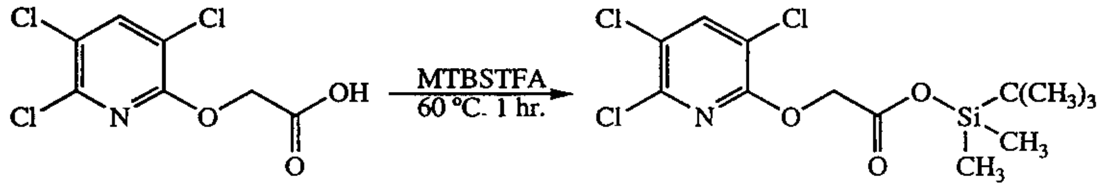
Table III. Recovery of 2-Methoxy-3,5,6-trichloropyridine from Fish Tissues

Sample Number	Date of Analysis	2-MP, µg/g Added	2-MP, µg/g Found	Percent Recovery	Statistical Calculations
SN17993002[a]	26-Jun-1997	0.000	0.000	--	
SN17993003[b]	3-Jul-1997	0.000	0.000	--	
SN18003302[c]	26-Jun-1997	0.000	0 003	--	
SN18003303[d]	3-Jul-1997	0.000	0.003	--	
SN20178201[e]	26-Jun-1997	0.000	0 000	--	
SN20178202[f]	3-Jul-1997	0.000	0.000	--	
SN17993002	26-Jun-1997	0.003	< 0 010	NA[g]	
SN17993003	3-Jul-1997	0.003	< 0.010	NA	
SN18003302	26-Jun-1997	0.003	< 0 010	NA	
SN18003303	3-Jul-1997	0.003	< 0.010	NA	
SN20178201	26-Jun-1997	0.003	< 0.010	NA	
SN20178202	3-Jul-1997	0.003	< 0.010	NA	
SN17993002	26-Jun-1997	0.010	0.0086	86	
SN17993002	26-Jun-1997	0.010	0.0089	89	
SN17993003	3-Jul-1997	0.010	0 0089	89	
SN17993003	3-Jul-1997	0.010	0.0095	95	
SN18003302	26-Jun-1997	0.010	0.0101	101	
SN18003302	26-Jun-1997	0.010	0.0098	98	
SN18003303	3-Jul-1997	0.010	0.0091	91	
SN18003303	3-Jul-1997	0.010	0 0091	91	$\bar{x}$ = 0.0093
SN20178201	26-Jun-1997	0.010	0 0082	82	s = 0.0007
SN20178201	26-Jun-1997	0.010	0.0086	86	$(3s)^h$ = 0.0021
SN20178202	3-Jul-1997	0.010	0.0098	98	$(10s)^i$ = 0.0069
SN20178202	3-Jul-1997	0.010	0.0105	105	RSD = 7.5%
SN17993002	26-Jun-1997	0.050	0.0437	87	
SN17993003	3-Jul-1997	0.050	0.0439	88	
SN18003302	26-Jun-1997	0.050	0.0492	98	
SN18003303	3-Jul-1997	0.050	0 0551	110	$\bar{x}$ = 0.0468
SN20178201	26-Jun-1997	0 050	0 0430	86	s = 0.0046
SN20178202	3-Jul-1997	0.050	0 0458	92	RSD = 9.9%
SN17993002	26-Jun-1997	0.500	0.399	80	
SN17993003	3-Jul-1997	0.500	0.497	99	
SN18003302	26-Jun-1997	0.500	0.461	92	
SN18003303	3-Jul-1997	0.500	0 478	96	$\bar{x}$ = 0.442
SN20178201	26-Jun-1997	0.500	0.383	77	s = 0 045
SN20178202	3-Jul-1997	0.500	0.432	86	RSD = 10.2%

Table III. (Cont.) Recovery of 2-Methoxy-3,5,6-trichloropyridine from Fish Tissues

Sample Number	Date of Analysis	2-MP, µg/g Added	2-MP, µg/g Found	Percent Recovery	Statistical Calculations
SN17993002	26-Jun-1997	5.00	4.103	82	
SN17993003	3-Jul-1997	5.00	5.066	101	
SN18003302	26-Jun-1997	5.00	4.580	92	
SN18003303	3-Jul-1997	5.00	4.968	99	$\bar{x}$ = 4.578
SN20178201	26-Jun-1997	5.00	4.035	81	s = 0.431
SN20178202	3-Jul-1997	5.00	4.717	94	RSD = 9.4%
				$\bar{x}$ = 92	
				s = 8	
				n = 30	

[a] SN17993002—Bluegill edible tissue.
[b] SN17993003—Bluegill inedible tissue.
[c] SN18003302—Catfish edible tissue. Recovery results from samples prepared using this sample were corrected for the amount of 2-MP found in the control.
[d] SN18003303—Catfish inedible tissue. Recovery results from samples prepared using this sample were corrected for the amount of 2-MP found in the control.
[e] SN20178201—Crayfish edible tissue.
[f] SN20178202—Crayfish inedible tissue.
[g] NA = not applicable. The residue was below the 0.010-µg/g limit of quantitation.
[h] Calculated limit of detection
[i] Calculated limit of quantitation.

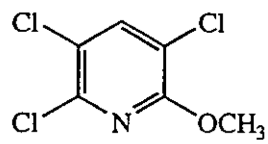


Triclopyr
Formula: $C_7H_4Cl_3NO_3$
Molecular Weight: 255

Triclopyr-TBDMS
Formula: $C_{13}H_{18}Cl_3NO_3Si$
Molecular Weight: 369

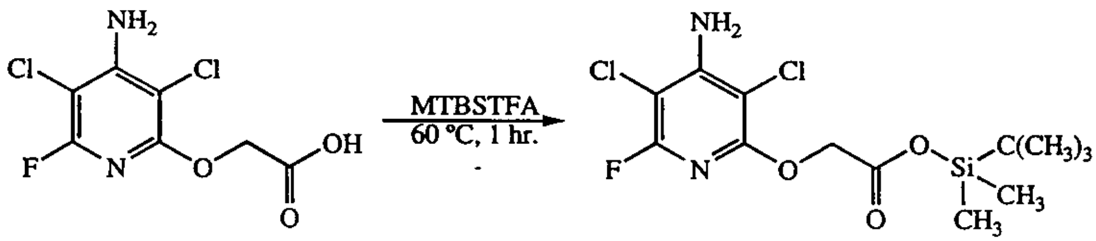
3,5,6-TCP
Formula: $C_5H_2Cl_3NO$
Molecular Weight: 197

3,5,6-TCP-TBDMS
Formula: $C_{11}H_{16}Cl_3NOSi$
Molecular Weight: 311



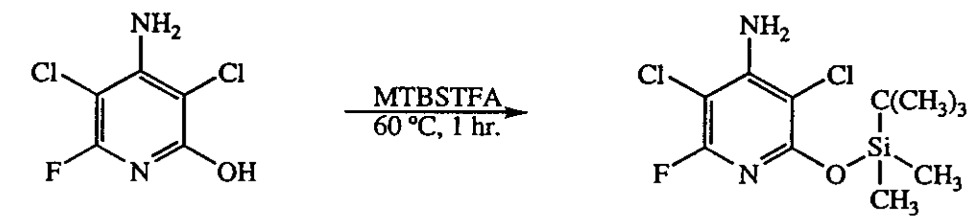
2-MP
Formula: $C_6H_4Cl_3NO$
Molecular Weight: 211

Figure 1. Chemical Structures of Triclopyr, 3,5,6-Trichloro-2-pyridinol and their TBDMS Derivatives, and 2-Methoxy-3,5,6-trichloropyridine



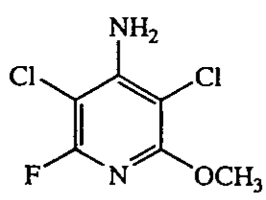
Fluroxypyr
Formula: $C_9H_5Cl_2FN_2O_3$
Molecular Weight: 254

Fluroxypyr-TBDMS
Formula: $C_{13}H_{19}Cl_2FN_2O_3Si$
Molecular Weight: 368



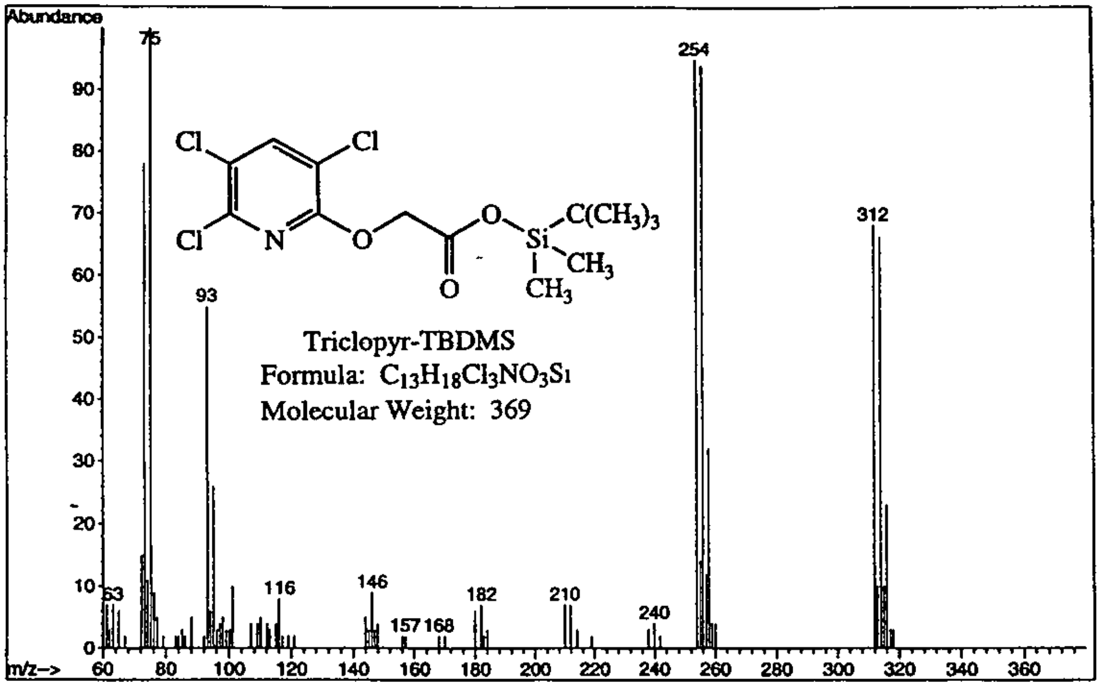
Fluroxypyr-DCP
Formula: $C_5H_3Cl_2FN_2O$
Molecular Weight: 196

Fluroxypyr-DCP-TBDMS
Formula: $C_{11}H_{17}Cl_2FN_2OSi$
Molecular Weight: 310



Fluroxypyr-MP
Formula: $C_6H_5Cl_2FN_2O$
Molecular Weight: 210

Figure 2. Chemical Structures of Fluroxypyr, 4-Amino-3,5-dichloro-6-fluoro-2-pyridinol and their TBDMS Derivatives, and 4-Amino-3,5-dichloro-6-fluoro-2-methoxypyridine



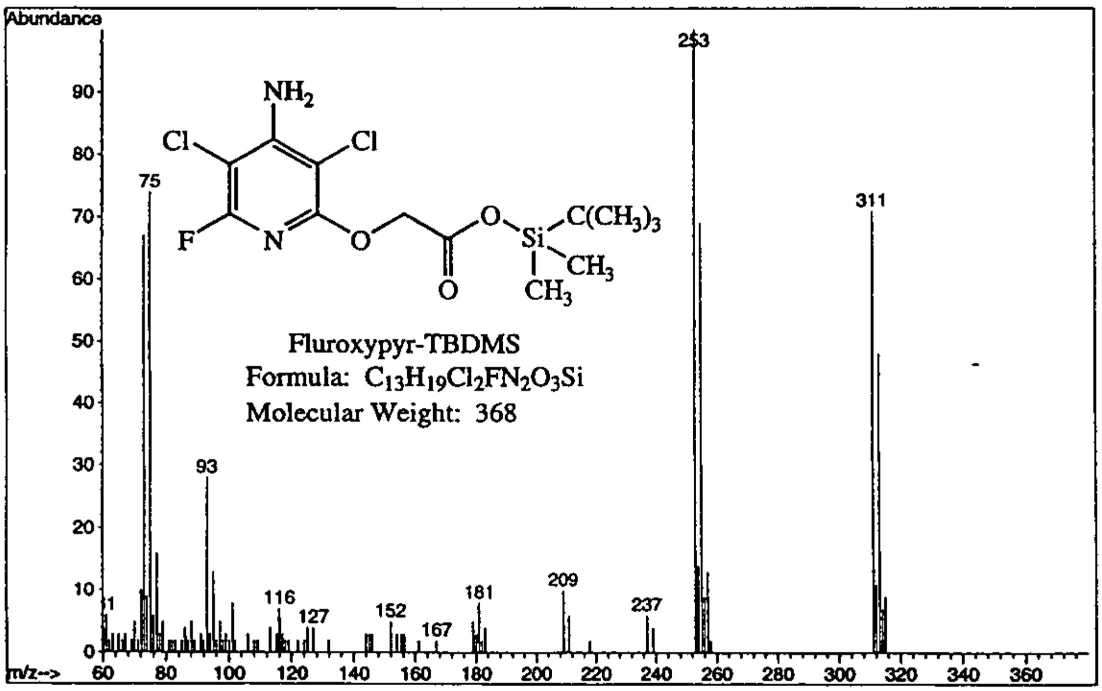
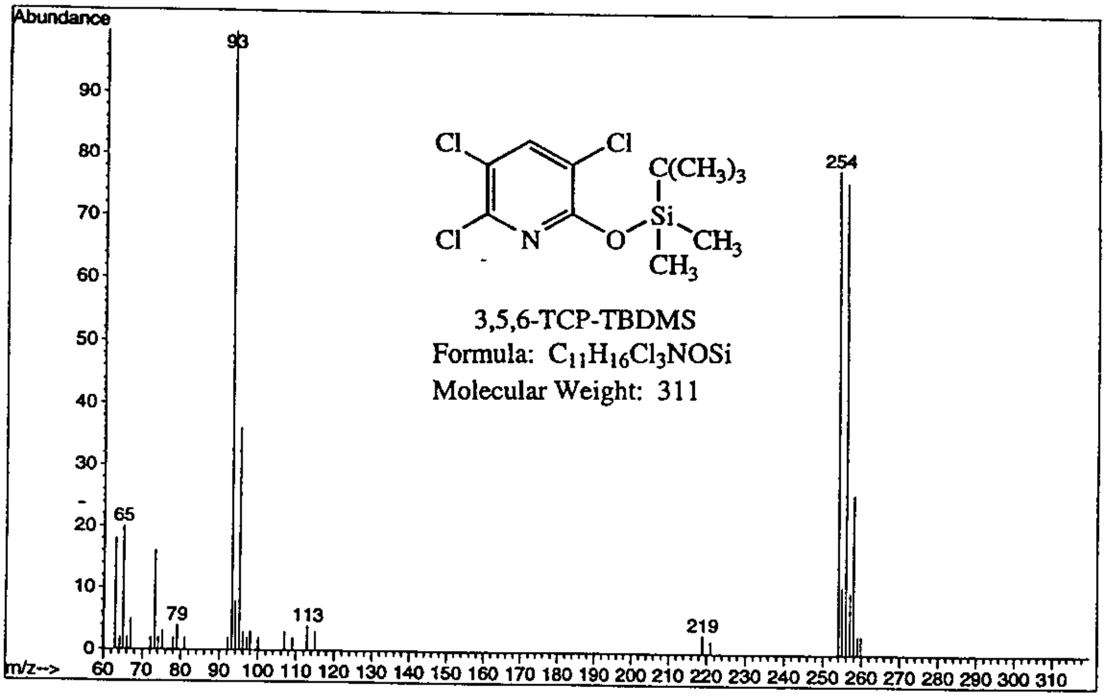


Figure 3. Mass Spectra of the TBDMS Derivatives of Triclopyr and Fluroxypyr



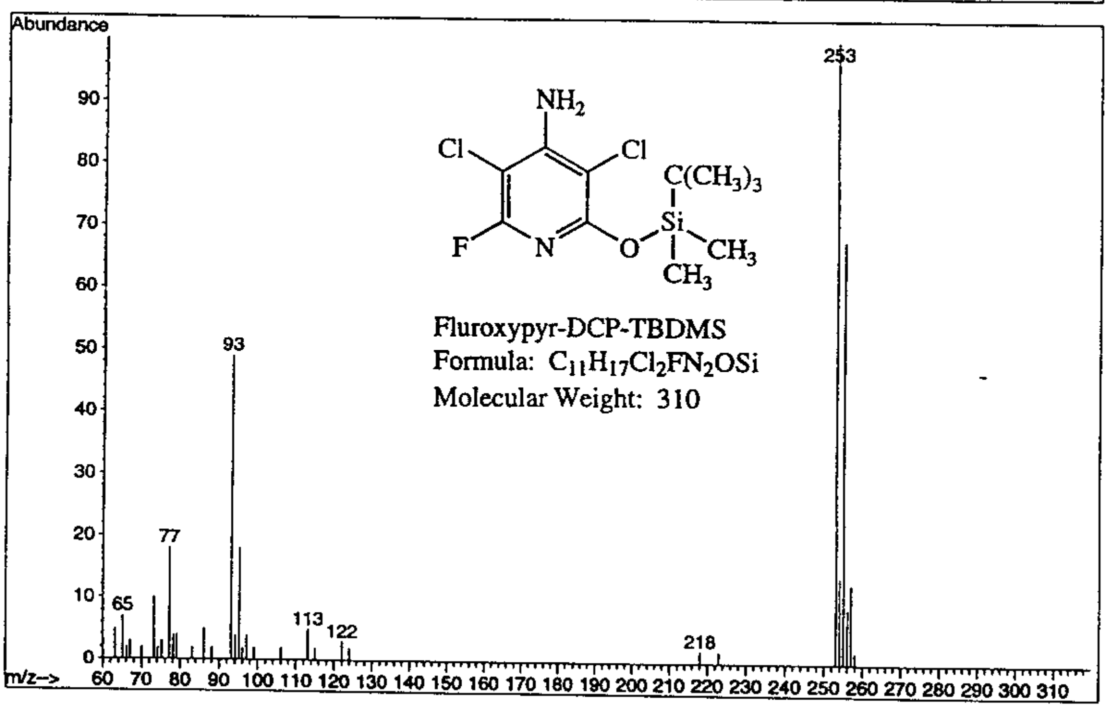
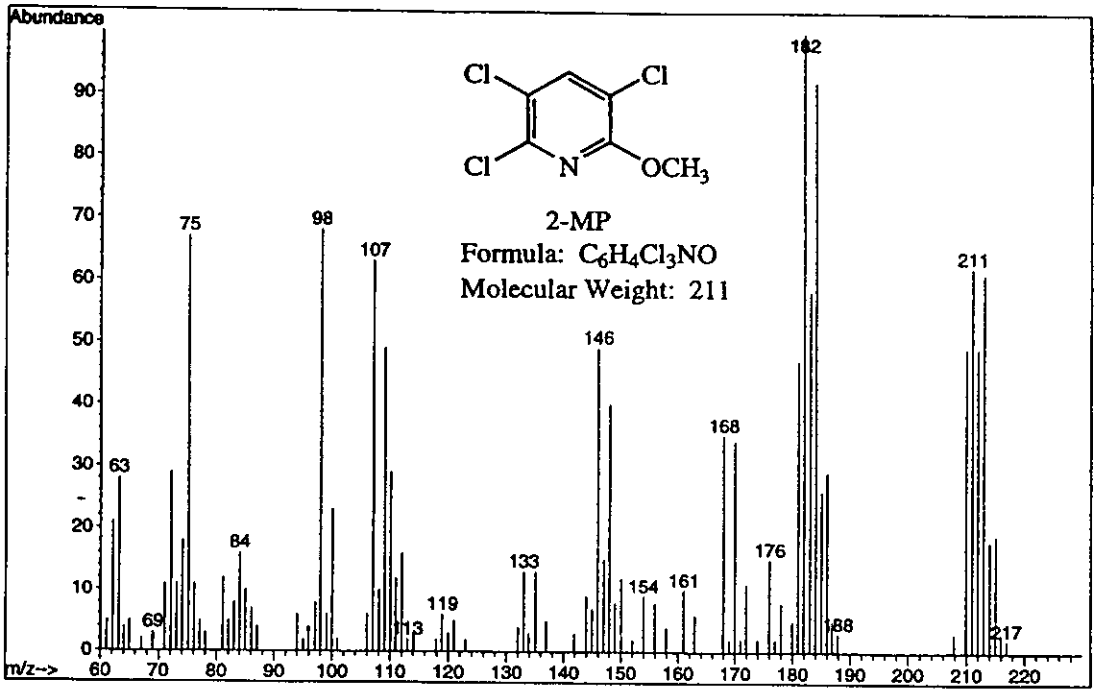


Figure 4. Mass Spectra of the TBDMS Derivatives of 3,5,6-Trichloro-2-pyridinol and 4-Amino-3,5-dichloro-6-fluoro-2-pyridinol



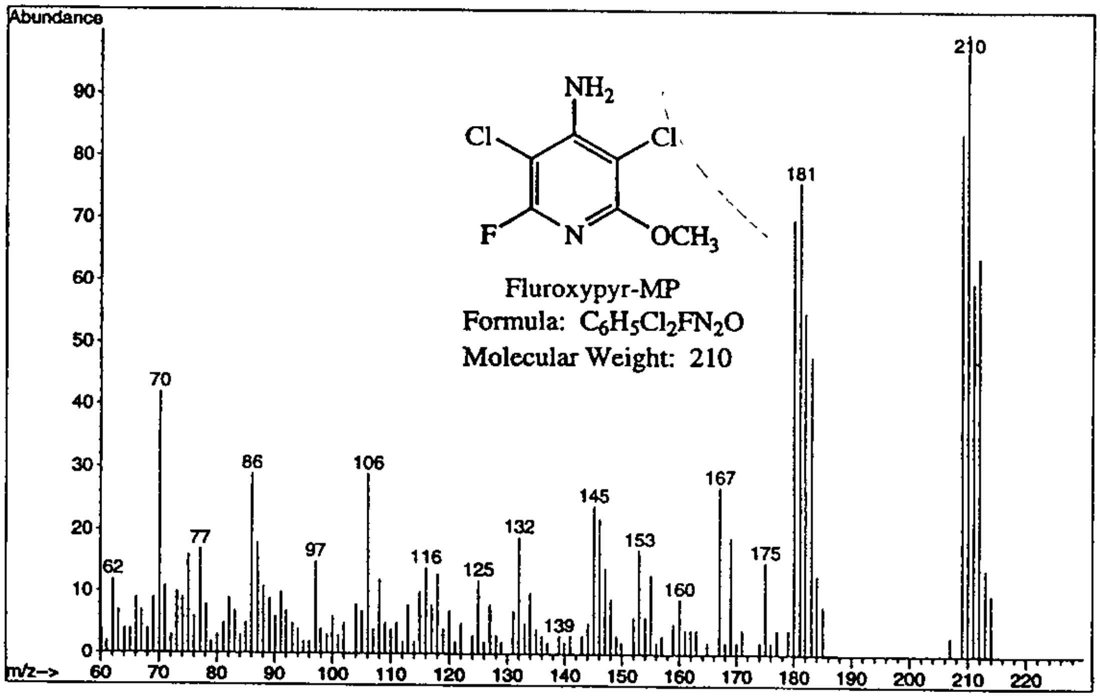
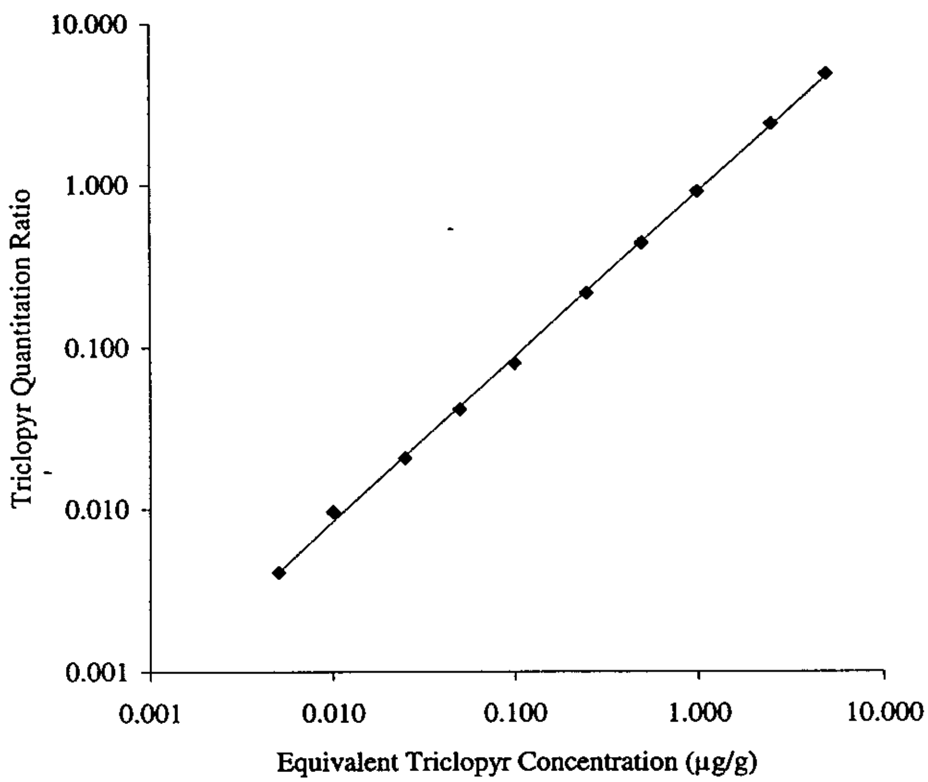


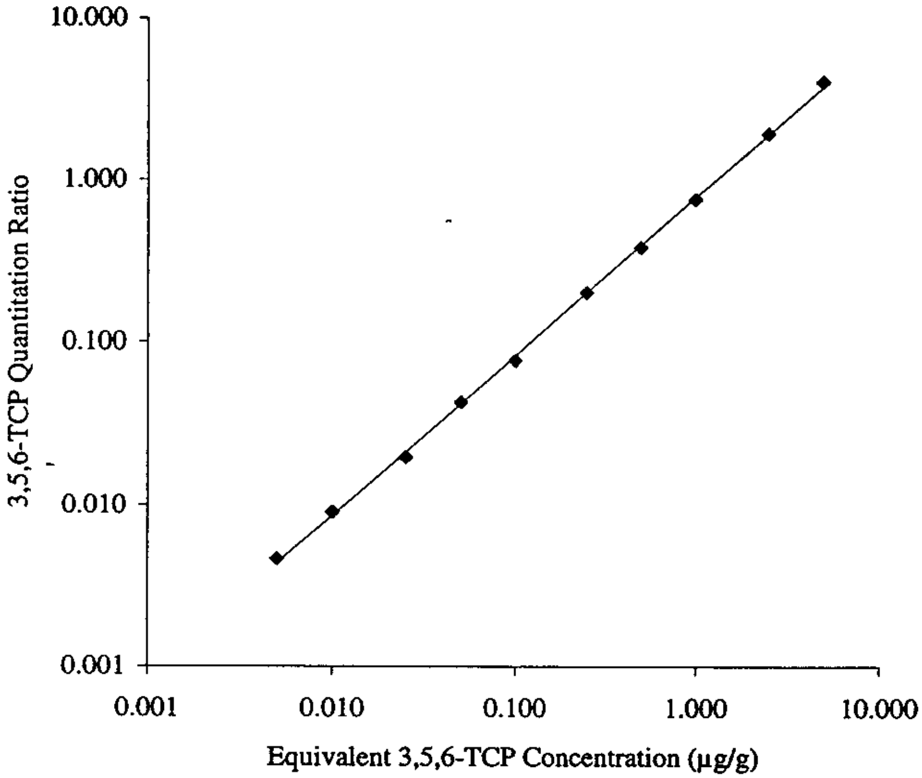
Figure 5. Mass Spectra of 2-Methoxy-3,5,6-trichloropyridine and 4-Amino-3,5-dichloro-6-fluoro-2-methoxypyridine



Triclopyr Concentration µg/mL	Equivalent Sample Conc. µg/g	Triclopyr Quantitation Ratio *m/z* 314 / *m/z* 311
0.005	0.005	0.00410
0.010	0.010	0.00961
0.025	0.025	0.02052
0.050	0.050	0.04120
0.100	0.100	0.07899
0.250	0.250	0.21348
0.500	0.500	0.43397
1.00	1.00	0.89599
2.50	2.50	2.33394
5.00	5.00	4.74820

Power Regression Equation: X = (Y/0.8925)^(1/1.0139)
Correlation Coefficient (r^2): 0.99934

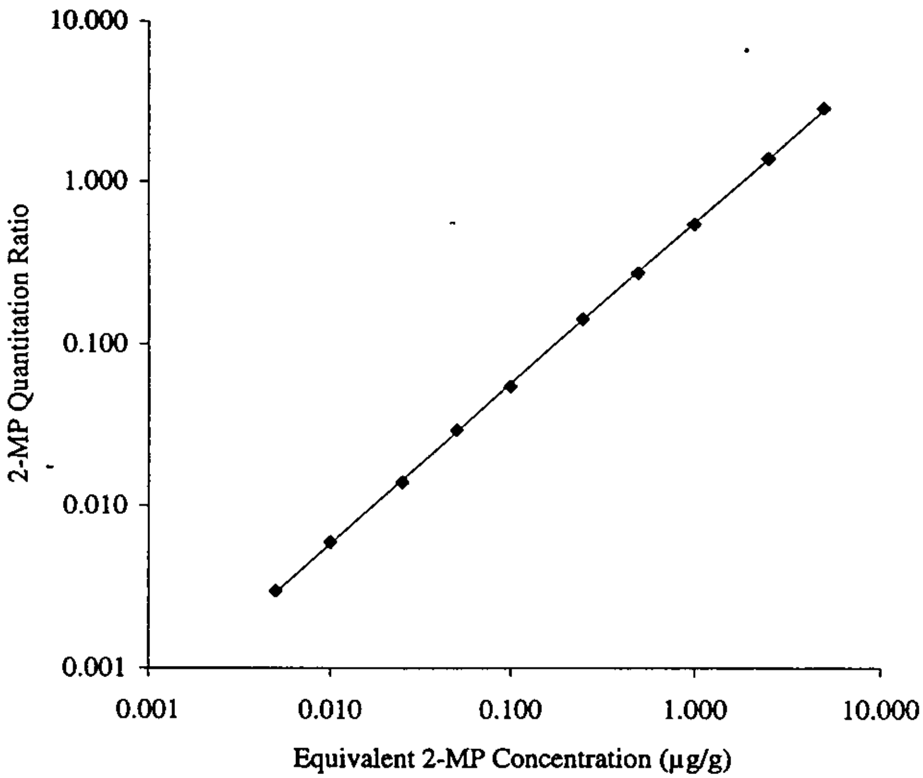
Figure 6. Typical Calibration Curve for the Determination of Triclopyr in Fish Tissues



3,5,6-TCP Concentration	Equivalent Sample Conc.	3,5,6-TCP Quantitation Ratio
µg/mL	µg/g	*m/z* 254 / *m/z* 253
0.005	0.005	0.00466
0.010	0.010	0.00904
0.025	0.025	0.01964
0.050	0.050	0.04240
0.100	0.100	0.07636
0.250	0.250	0.19917
0.500	0.500	0.38024
1.00	1.00	0.74854
2.50	2.50	1.90305
5.00	5.00	3.99394

Power Regression Equation: X = (Y/0.7720)^(1/0.9755)
Correlation Coefficient (r^2): 0.99950

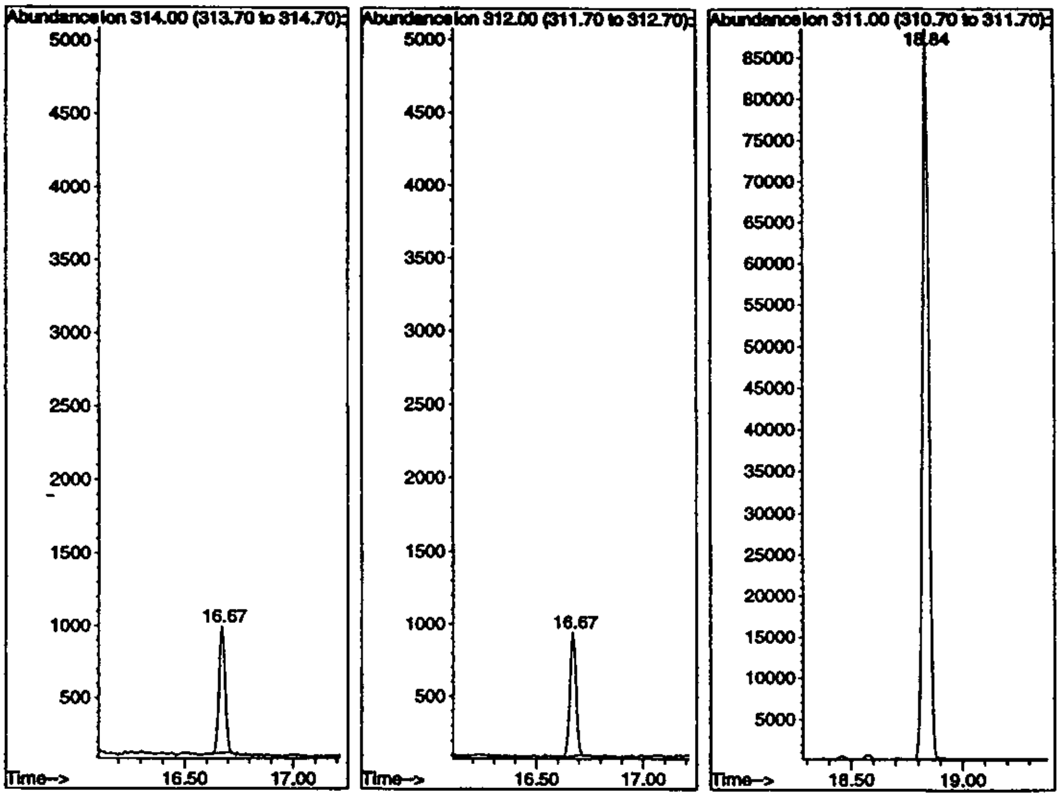
Figure 7. **Typical Calibration Curve for the Determination of 3,5,6-Trichloro-2-pyridinol in Fish Tissues**



2-MP Concentration µg/mL	Equivalent Sample Conc. µg/g	2-MP Quantitation Ratio *m/z* 213 / *m/z* 210
0.005	0.005	0.00298
0.010	0.010	0.00594
0.025	0.025	0.01391
0.050	0.050	0.02918
0.100	0.100	0.05408
0.250	0.250	0.14065
0.500	0.500	0.27228
1.00	1.00	0.54151
2.50	2.50	1.37561
5.00	5 00	2.77943

Power Regression Equation: X = (Y/0.5504)^(1/0.9886)
Correlation Coefficient (r^2): 0.99988

Figure 8. Typical Calibration Curve for the Determination of 2-Methoxy-3,5,6-trichloropyridine in Fish Tissues




```
Data File   : 0601006.D
ALS Bottle  : 6
Date        :  3 Jul 97  18:27
Data Path   : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument  : GC/MSD S/N 3307A00401
Protocol    : RES94084

Sample Name: Triclopyr Standard - 0010 ng/mL
Sample Info: Equivalent to 0010 ng/g in fish
Operator   : Edward L. Olberding

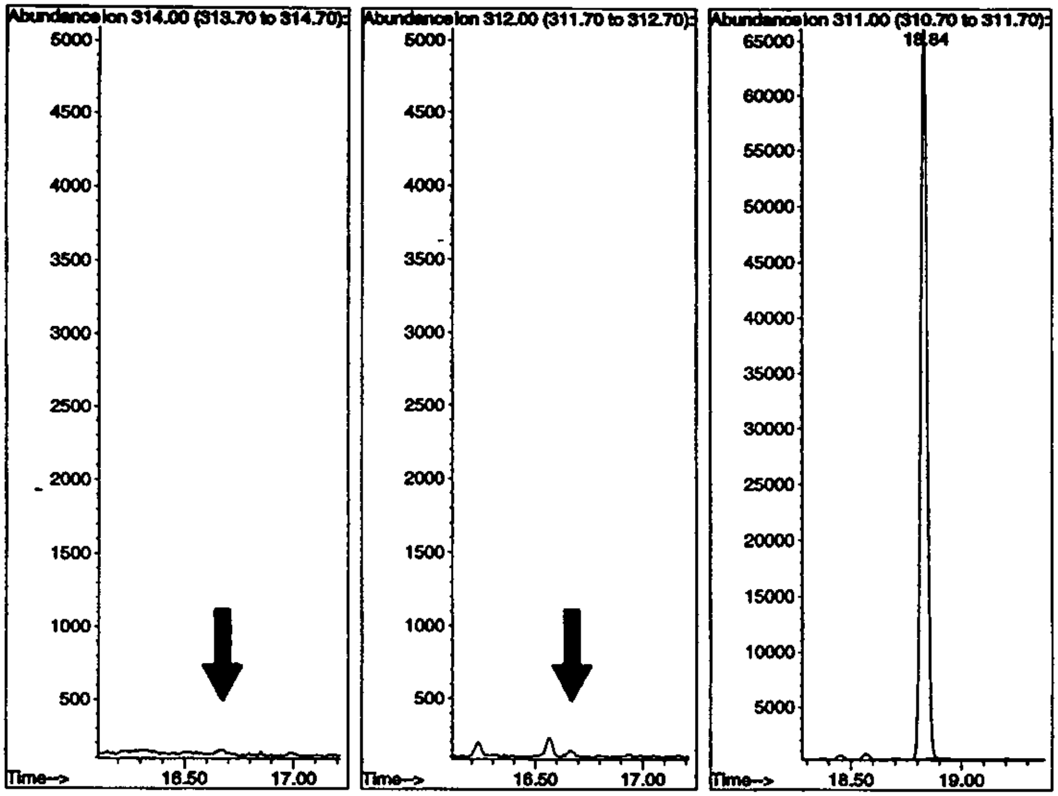
INTERNAL STANDARD RETENTION TIME:    18.84
     PEAK AREA (M/Z 311)        :  1723923

TRICLOPYR RETENTION TIME        :    16.67
     PEAK AREA (M/Z 314)        :    16562
     PEAK AREA (M/Z 312)        :    16597
```

Equivalent Triclopyr Concentration: 0.0100 µg/g

Average Confirmation Ratio: 0.9948

Figure 9. Typical Chromatogram of a 0.010-µg/mL Standard Equivalent to 0.010 µg/g of Triclopyr in Fish Tissue

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Data File  : 1901019.D
ALS Bottle : 19
Date       :  4 Jul 97   1:05
Data Path  : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument : GC/MSD S/N 3307A00401
Protocol   : RES94084

Sample Name: SN20178202 - Control
Sample Info: Sample 26 - Crayfish - Inedible
Operator   : Edward L. Olberding

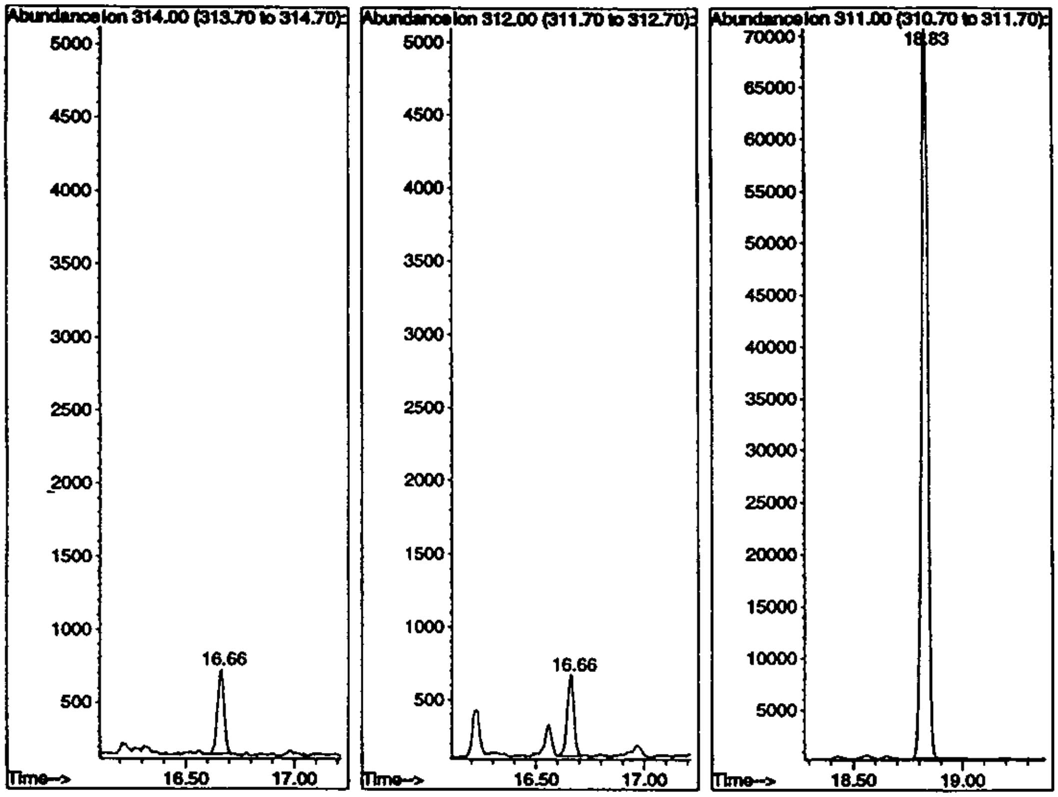
INTERNAL STANDARD RETENTION TIME:    18.84
     PEAK AREA (M/Z 311)        :  1323914

NO TRICLOPYR FOUND
```

Triclopyr Concentration: 0.0000 µg/g

Average Confirmation Ratio: 0.9948

Figure 10. Typical Chromatogram of a Control Crayfish Inedible Tissue Sample Containing No Detectable Residue of Triclopyr




```
Data File   : 2901029.D
ALS Bottle  : 29
Date        :  4 Jul 97   6:10
Data Path   : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument  : GC/MSD S/N 3307A00401
Protocol    : RES94084

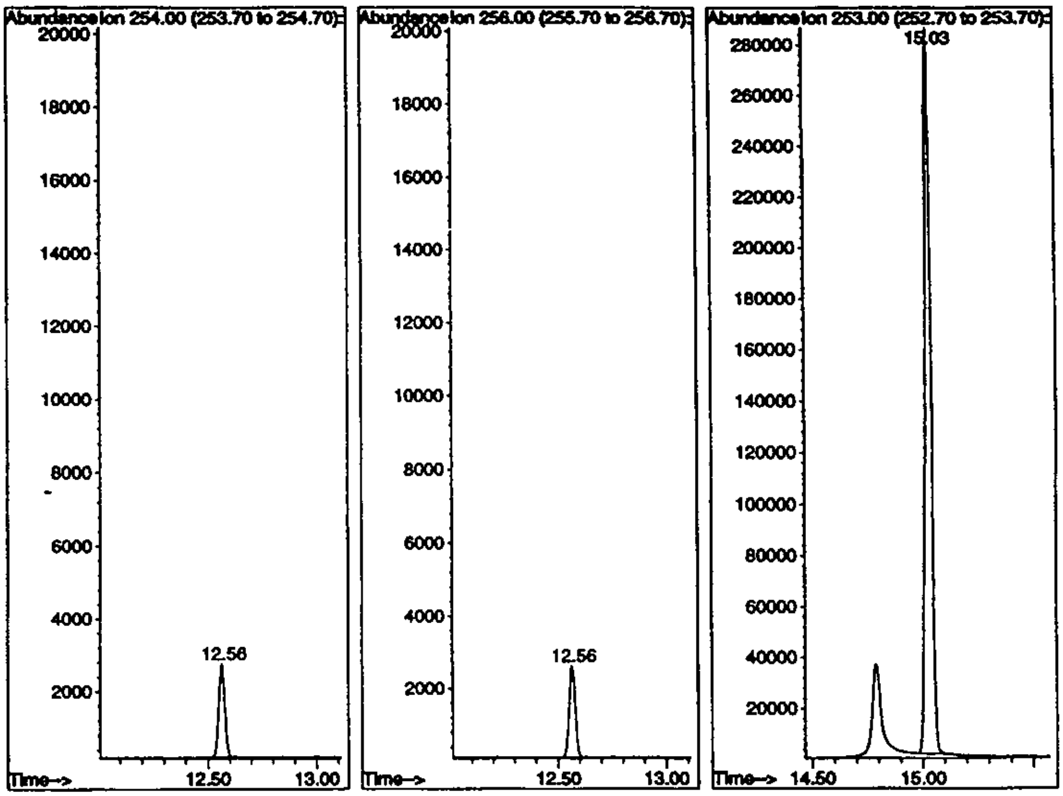
Sample Name: SN20178202 - Spiked at 0010 ng/g
Sample Info: Sample 34 - Crayfish - Inedible
Operator   : Edward L. Olberding


INTERNAL STANDARD RETENTION TIME:     18.83
     PEAK AREA (M/Z 311)        :   1443236

TRICLOPYR RETENTION TIME        :     16.66
     PEAK AREA (M/Z 314)        :     12260
     PEAK AREA (M/Z 312)        :     11953
```

Triclopyr Concentration: 0.0102 µg/g
Recovery: 102%
Average Confirmation Ratio: 0.9948

Figure 11. Typical Chromatogram of a Crayfish Inedible Tissue Sample Fortified with 0.010 µg/g of Triclopyr




```
Data File  : 0601006.D
ALS Bottle : 6
Date       :  3 Jul 97  18:27
Data Path  : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument : GC/MSD S/N 3307A00401
Protocol   : RES94084

Sample Name: Triclopyr Standard - 0010 ng/mL
Sample Info: Equivalent to 0010 ng/g in fish
Operator   : Edward L. Olberding

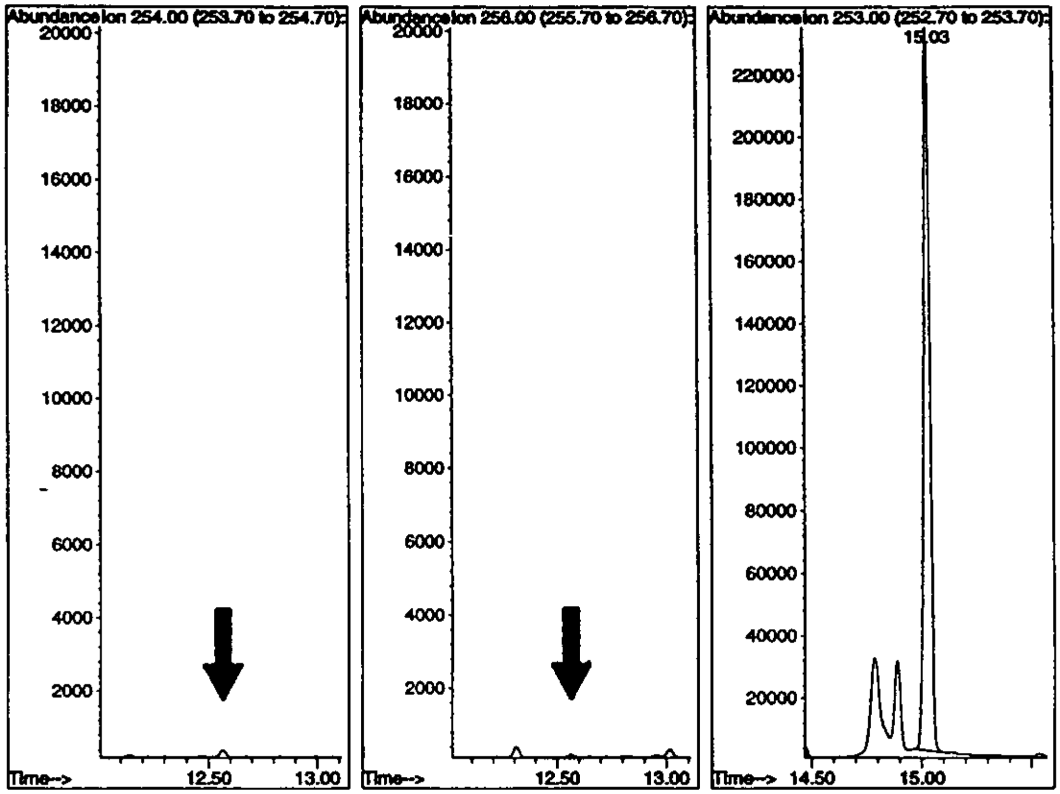

INTERNAL STANDARD RETENTION TIME:    15.03
     PEAK AREA (M/Z 253)         :  5444473

3,5,6-TCP RETENTION TIME         :    12.56
     PEAK AREA (M/Z 254)         :    49230
     PEAK AREA (M/Z 256)         :    48808
```

Equivalent 3,5,6-TCP Concentration: 0.0100 µg/g

Average Confirmation Ratio: 0.9906

Figure 12. Typical Chromatogram of a 0.010-µg/mL Standard Equivalent to 0.010 µg/g of 3,5,6-TCP in Fish Tissue




```
Data File  : 1901019.D
ALS Bottle : 19
Date       :  4 Jul 97   1:05
Data Path  : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument : GC/MSD S/N 3307A00401
Protocol   : RES94084

Sample Name: SN20178202 - Control
Sample Info: Sample 26 - Crayfish - Inedible
Operator   : Edward L. Olberding

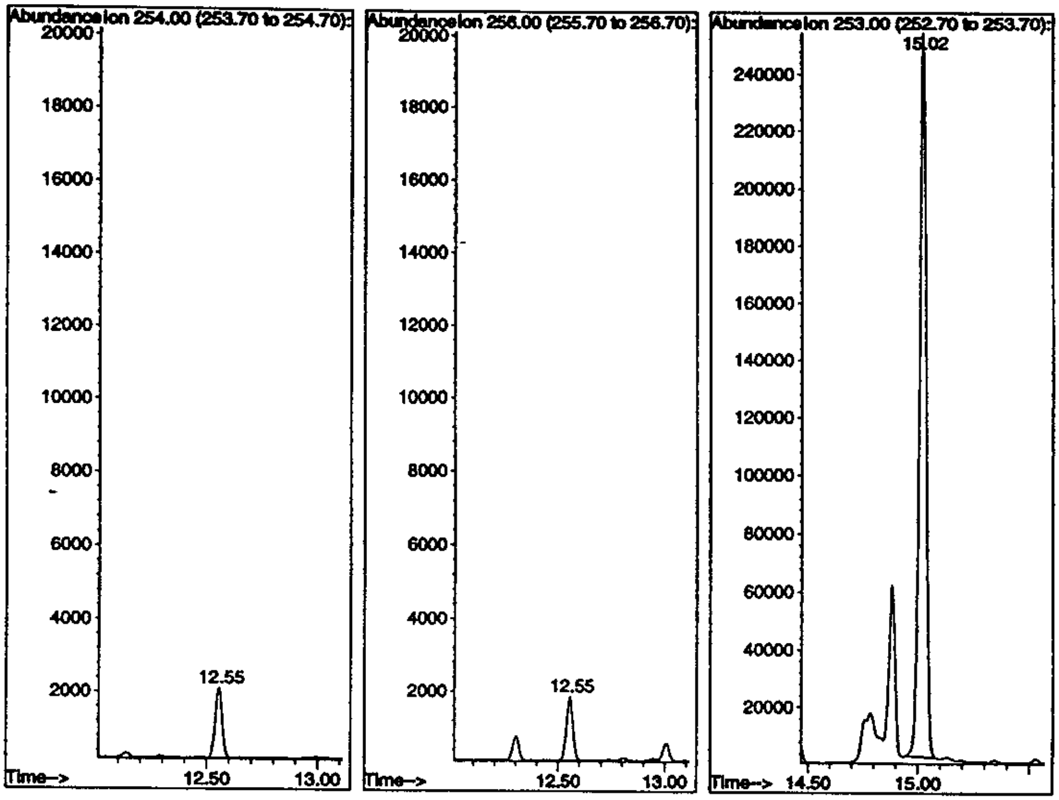

INTERNAL STANDARD RETENTION TIME:    15.03
     PEAK AREA (M/Z 253)        :  4486369

NO 3,5,6-TCP FOUND
```

3,5,6-TCP Concentration: 0.0000 µg/g

Average Confirmation Ratio: 0.9906

Figure 13. Typical Chromatogram of a Control Crayfish Inedible Tissue Sample Containing No Detectable Residue of 3,5,6-TCP




```
Data File  : 2901029.D
ALS Bottle : 29
Date       :  4 Jul 97   6:10
Data Path  : C:\HPCHEMPC\1\DATA\RES94084\A070397A.ELO\
Instrument : GC/MSD S/N 3307A00401
Protocol   : RES94084

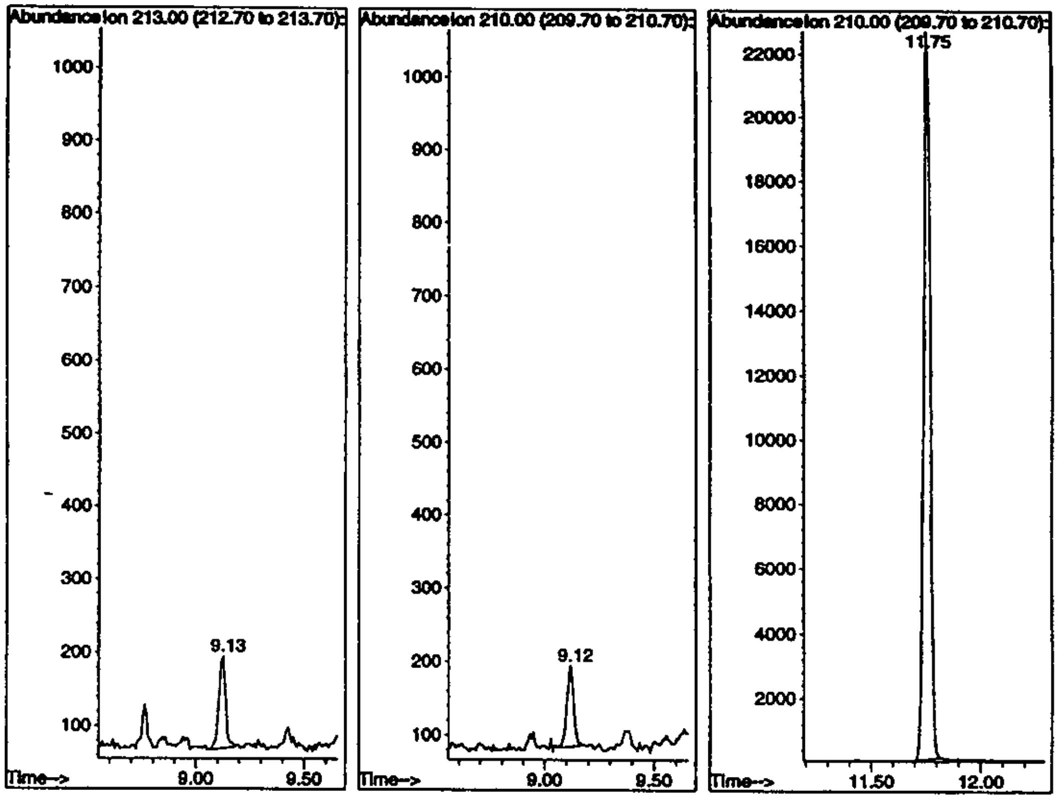
Sample Name: SN20178202 - Spiked at 0010 ng/g
Sample Info: Sample 34 - Crayfish - Inedible
Operator   : Edward L. Olberding


INTERNAL STANDARD RETENTION TIME:     15.02
     PEAK AREA (M/Z 253)        :   5192065

3,5,6-TCP RETENTION TIME        :     12.55
     PEAK AREA (M/Z 254)        :     41066
     PEAK AREA (M/Z 256)        :     36079
```

3,5,6-TCP Concentration: 0.0091 µg/g
Recovery: 91%
Average Confirmation Ratio: 0.9906

Figure 14. Typical Chromatogram of a Control Crayfish Inedible Tissue Sample Fortified with 0.010 µg/g of 3,5,6-TCP

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Data File   : 0601006.D
ALS Bottle  : 6
Date        :  5 Jul 97  12:20
Data Path   : C:\HPCHEMPC\1\DATA\RES94084\A070597A.ELO\
Instrument  : GC/MSD S/N 3307A00401
Protocol    : RES94084

Sample Name: Triclopyr Standard - 0010 ng/mL
Sample Info: Equivalent to 0010 ng/g in fish
Operator   : Edward L. Olberding

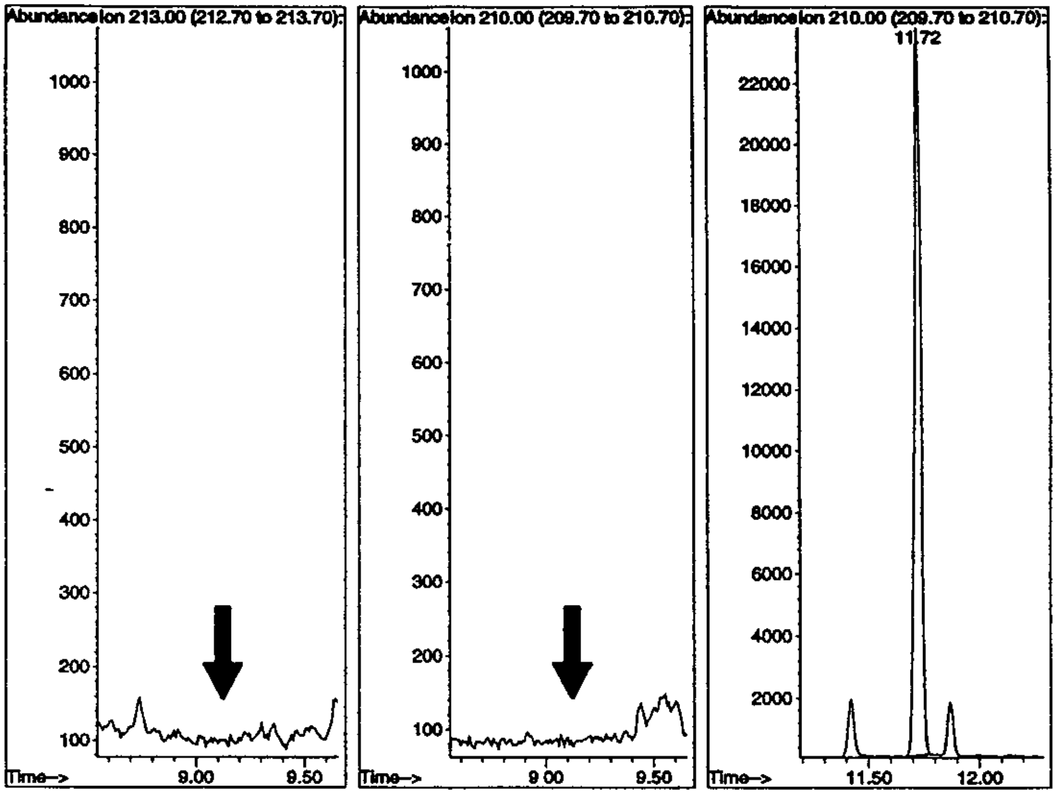
INTERNAL STANDARD RETENTION TIME:    11.75
     PEAK AREA (M/Z 210)        :   465618

2-MP RETENTION TIME             :     9.13
     PEAK AREA (M/Z 213)        :     2765
     PEAK AREA (M/Z 210)        :     2357
```

Equivalent 2-MP Concentration: 0.0100 µg/g

Average Confirmation Ratio: 0.8162

Figure 15. Typical Chromatogram of a 0.010-µg/mL Standard Equivalent to 0.010 µg/g of 2-MP in Fish Tissue




```
Data File   : 1901019.D
ALS Bottle  : 19
Date        :  5 Jul 97  18:04
Data Path   : C:\HPCHEMPC\1\DATA\RES94084\A070597A.ELO\
Instrument  : GC/MSD S/N 3307A00401
Protocol    : RES94084

Sample Name: SN20178202 - Control
Sample Info: Sample 26 - Crayfish - Inedible
Operator   : Edward L. Olberding

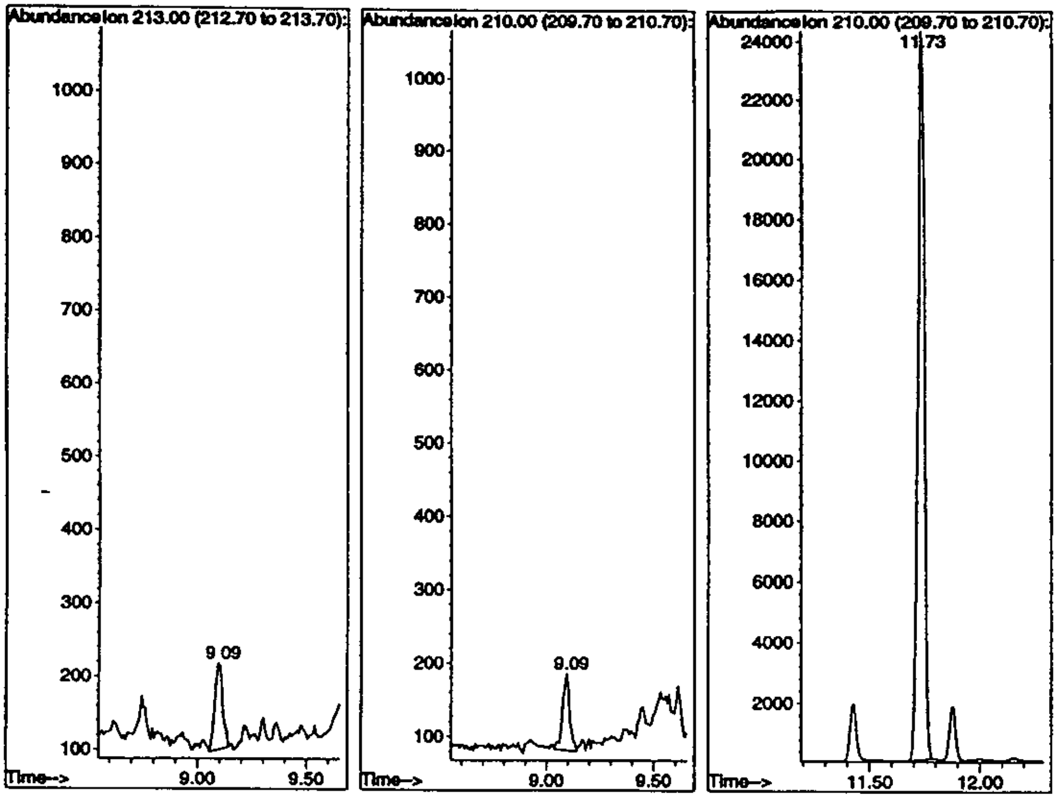
INTERNAL STANDARD RETENTION TIME:    11.72
     PEAK AREA (M/Z 210)        :   475764

NO 2-MP FOUND
```

2-MP Concentration: 0.0000 µg/g

Average Confirmation Ratio: 0.8162

Figure 16. Typical Chromatogram of a Control Crayfish Inedible Tissue Containing No Detectable Residue of 2-MP

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Data File   : 3001030.D
ALS Bottle  : 30
Date        :  5 Jul 97  22:56
Data Path   : C:\HPCHEMPC\1\DATA\RES94084\A070597A.ELO\
Instrument  : GC/MSD S/N 3307A00401
Protocol    : RES94084

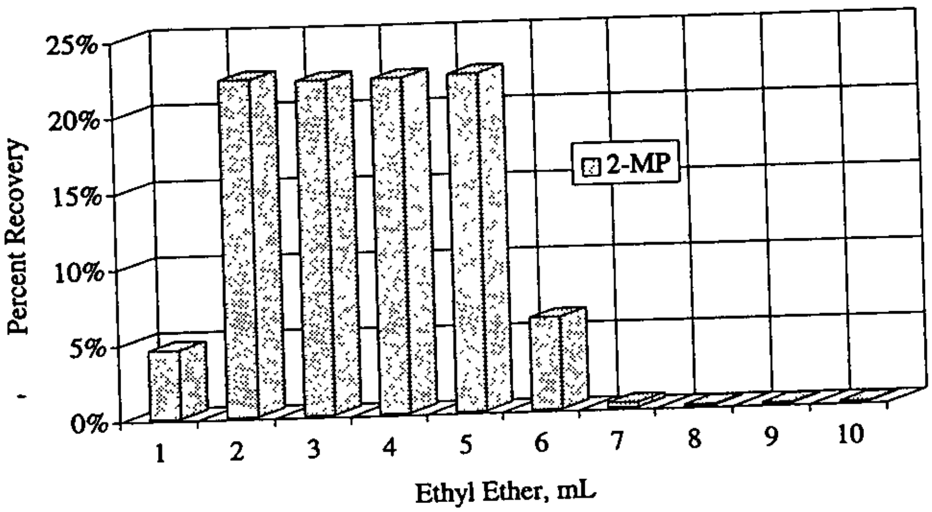
Sample Name: SN20178202 - Spiked at 0010 ng/g
Sample Info: Sample 35 - Crayfish - Inedible
Operator   : Edward L. Olberding

INTERNAL STANDARD RETENTION TIME:    11.73
     PEAK AREA (M/Z 210)        :   492706

2-MP RETENTION TIME             :     9.09
     PEAK AREA (M/Z 213)        :     3002
     PEAK AREA (M/Z 210)        :     2444
```

2-MP Concentration: 0.0102 µg/g
Recovery: 102%
Average Confirmation Ratio: 0.8162

Figure 17. Typical Chromatogram of a Control Crayfish Inedible Tissue Fortified with 0.010 µg/g of 2-MP



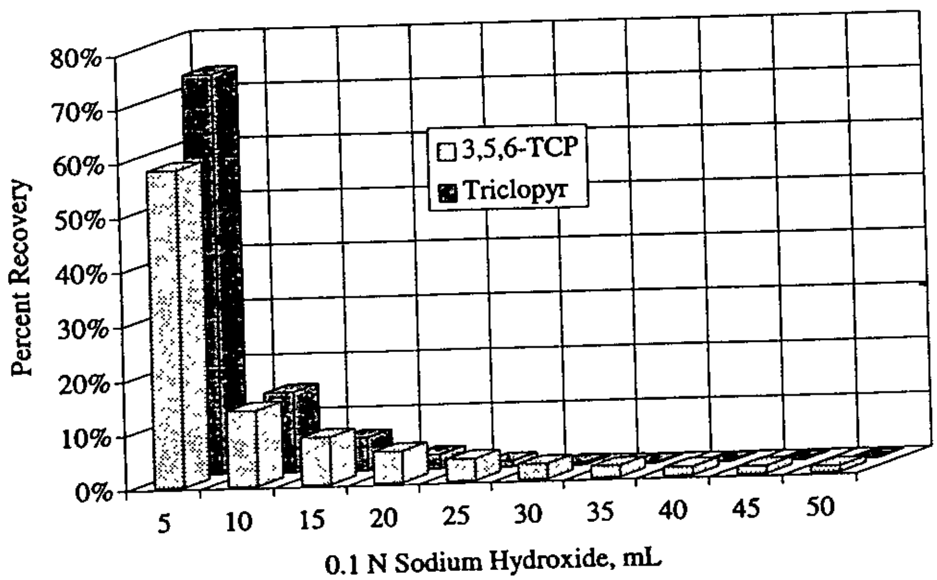
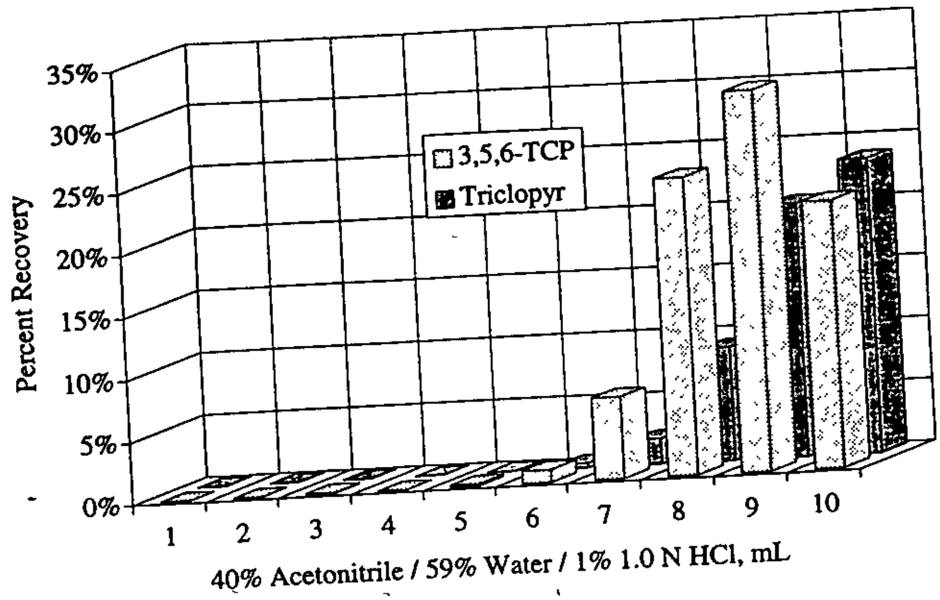


Figure 18. Typical Alumina SPE Elution Profiles for 2-Methoxy-3,5,6-trichloropyridine (Ethyl Ether) and Triclopyr and 3,5,6-Trichloro-2-pyridinol (0.1 N Sodium Hydroxide)



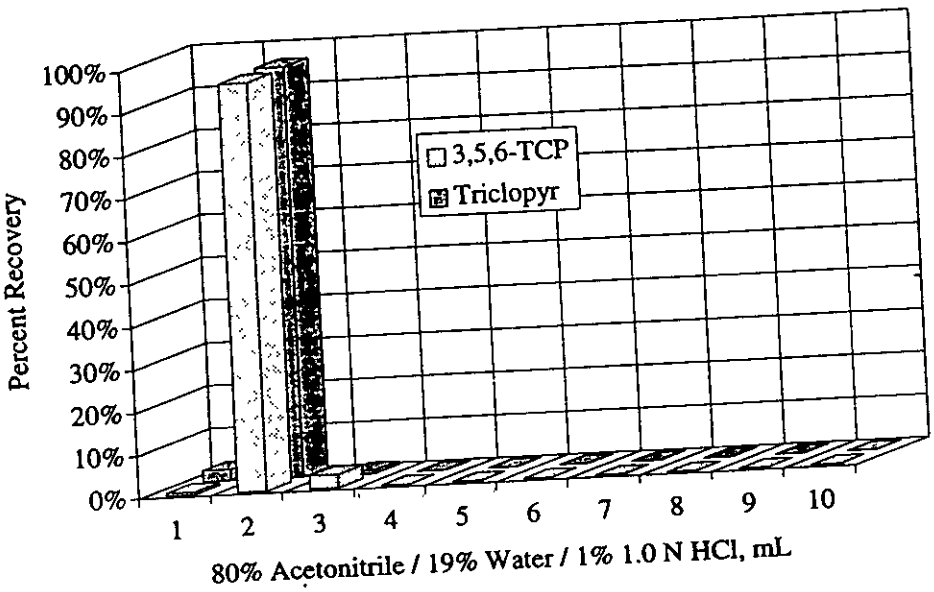


Figure 19. Typical C_{18} SPE Elution Profiles for Triclopyr and 3,5,6-Trichloro-2-pyridinol

Conditions and Substitutions used by ACB

1. The ACB used the following instrumentation: HP model 5890 Series II plus gas chromatograph, HP model 5972 Mass Selective Detector ACB used a Restek Rtx-1701 fused silica capillary column, 10 m x 0.18 mm i.d , 0.4 µm film thickness (Cat. # 42010). The temperature program was the same as the petitioner: 70 °C for 1.0 min, 70 °C to 255 °C at 10 °C/min, 255 C to 280 °C at 20 °C/min, and 280 °C for 4.75 min. The injector and interface temperatures were 280 °C The carrier gas was helium. The injection volume was 1 µL.

2. The ACB purchased graduated 10 mL Reacti-Vials™ from Pierce Chemical Co , Rockford, Illinois 61105 (Cat.#13225) for the final extraction step. This substitution eliminated the need to estimate the 1 mL final volume in the 12 mL unmarked vial used in the method.

3a. The method stated to profile each lot of SPE columns prior to use ACB found only one lot of Supelco, Inc. (the method calls for Bakerbond spe™) C_{18} columns that would give adequate recoveries to use in the trial. The method addresses the use of equivalent reagents with the understanding that they must be confirmed by appropriate tests The ACB contacted the petitioner to discuss the low recovers from the profiling results. The petitioner suggested the use of other manufacturers of C_{18} columns. In addition, the petitioner gave the ACB permission to increase the percentage of acetonitrile (ACN) in the elution solution to increase recoveries. The ACB used an elution solution: ACN-H_2O-1N HCl (90+9+1, v/v/v). The method used ACN-H_2O-1N HCl (80+19+1, v/v/v). The ACB suggests that a column profile be performed using a standard that mirrors the tolerance level as well as the level (1µg/mL) in the profiling instructions.

3b. The ACB found that 5 mL of elution solution was a more efficient volume than the 4 mL used in the method (step I.1.y.(8)). In addition, the ACB collected a second elution volume (5 mL) in another 40 mL vial and stored it capped in the refrigerator. This could be worked up later if the recoveries were low (<70%) for the first aliquot. This is a recommendation for higher tolerance levels

4 The method did not give storage or stability data for the standard solutions or the derivatized calibration standards. The ACB stored stock standards and derivatized calibration standards in the refrigerator at 2-11°C. The ACB found that the final extracts were stable at room temperature for a least 48 hours in capped auto-sampler vials.

5. The method emphasizes, in a note (L.9), that it is critical not to transfer any water over with the 1-chlorobutane layer because it will have a deleterious effect on the derivatization and subsequent GC/MSD analysis. The ACB observed as much as a ten fold difference in area responses when aqueous (salt) was in the final extract.

6 The method suggested the use of a calibration exponential regression equation to determine sample concentrations (quantitation ratio vs concentration). The ACB ran calibration curves to demonstrate linearity over the dynamic range of the fortified samples. The ACB used the standard regression equation (Y = mx + b) In this method validation, The ACB determined sample concentrations from a ratio of sample responses to the average of standard responses that bracketed the samples during the analysis